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13. SUPPLEMENTARY NOTES

14. ABSTRACT

To define the mechanisms involved in prostate cancer progression, we have found that molecular chaperones are elevated, causing increased telomerase activity. In order to determine the importance of the chaperones during prostate cancer progression, we proposed 2 specific aims: 1-define whether ectopic chaperone expression results in transformation, and 2-determine whether chaperones are targets for prostate cancer therapy. The hsf-1 transcription factor was over-expressed in non-tumorigenic prostate cells, resulting in increased hsp90 and hsp70 expression, an upregulation of telomerase through a global chaperone increase, and no effect on tumorigenicity. Using both a pharmacologic (radicicol) and genetic (siRNA) approaches, depletion of functional Hsp90 in prostate cancer cells caused dramatic telomere shortening followed by apoptosis. Of particular significance, these cells exhibit a high level of nitric oxide synthase (NOS)-dependent free radical production, and simultaneous treatment of cells with the NOS inhibitor L-NAME resulted in telomere elongation and prevention of apoptosis. In addition, we observe significant DNA damage assessed by telomere dysfunction, although in the absence of a classical DNA damage response. Overall, our data suggest a novel mechanism whereby inhibition of Hsp90 disrupts free radical homeostasis and contributes directly to telomere erosion, further implicating Hsp90 as a potential therapeutic target for prostate cancer.

15. SUBJECT TERMS
telomerase, chaperone, tumors, transformation, hsp90, radicicol

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Introduction

We have previously focused on the chaperone complex and its regulation of telomerase during prostate cancer progression. We initially showed the telomerase is upregulated during progression and that this elevated telomerase activity is due to increased chaperone-mediated assembly rather than transcription (Akalin et al., 2001). We have additionally shown that the hsp90 and p23 chaperones remain associated with the active enzyme, which is unique for hsp90 targets (Forsythe et al., 2001) (Figure 1). Our goal for the current project, funded by the DOD, was to determine if chaperone over-expression is sufficient for transformation and if chaperones are adequate targets for prostate cancer cells. As in our previous report, we have essentially accomplished these tasks in vitro, meaning that no in vivo tumorigenesis data has been obtained. Our goal for this final report is to define what has been done in the last year of no cost extended funding, much of which has been characterization and publication generation.

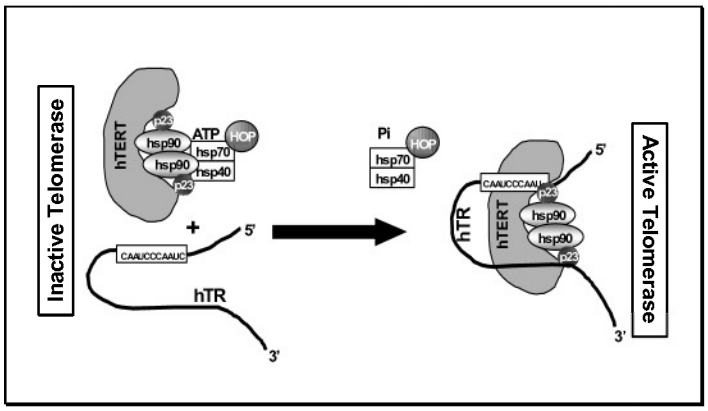

Figure 1. The hsp90 complex is required for assembly of active telomerase. Our working model for the chaperone-mediated ordered assembly of active human telomerase. [hTR - human telomerase RNA; hTERT - human telomerase reverse transcriptase]. Taken from Forsythe et al., 2001.

Body

Before we begin with a discussion of our most recent data, it is necessary to understand the in vitro model system used for all of these studies. This model is unique in that it has untransformed, transformed, and metastatic sublines all with a genetically similar background (Figure 2), making genetic and genomic changes more tractable and biologically relevant to the study of prostate cancer. Normal human prostate epithelial cells were immortalized by expression with the SV40 large T antigen oncogene, and an immortal, non-tumorigenic, telomerase-positive cell line was selected, P69. When injected into nude mice, no tumors formed within the standard 8-12 weeks, but if left in the animal for 6 months, 2 palpable sporadic tumors from a total of 19 mice formed after in vivo selection (Bae et al., 1994) (Figure 2). Both lines, M2205 and M2182, were propagated in culture and reinjected into mice and were found to be tumorigenic. After undergoing another round of selection, the metastatic subline, M12, was generated. As our model for prostate cancer, the P69-M12 progression scheme provides an excellent system from a defined genetic background to study the molecular and cellular changes that occur during prostate cancer progression. Having found elevated telomerase levels in the more advanced prostate cancer lines and tumor samples (Akalin et al., 2001), our data indicates that this change in activity is due to an increase chaperone-mediated telomerase assembly rather than expression of the hTERT and hTR core components of the telomerase holoenzyme. As such, our goals are to determine if chaperones are the cause of the transformation event during prostate cancer progression and to show that chaperones are likely targets for anti-telomerase therapy in advanced prostate cancer.

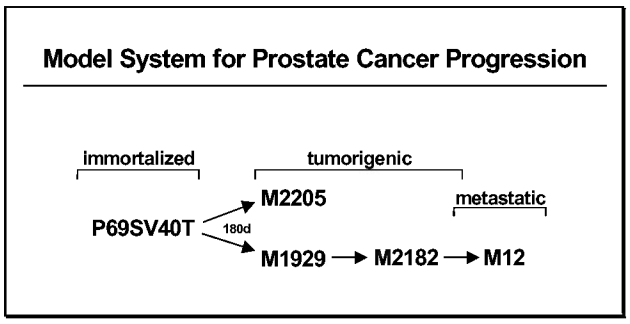

Figure 2. Prostate cancer progression model system. In our *in vitro* model of prostate cancer progression, as cells convert from immortal to tumorigenic and eventually metastatic capabilities, both telomerase activity and global chaperone protein levels increase. Taken from Akalin et al., 2001.

***Specific Aim #1*: Define the role of chaperones and telomerase activation during prostate cancer progression.**

This first aim is designed to determine if the elevated chaperone expression levels can cause transformation in the non-tumorigenic P69 cells. We have designed and made retroviral constructs for chaperone-related genes, hTERT, and oncogenic ras. The expression of these constructs and gene was discussed in the report last year, and specific points are discussed below without the figures from last year. 2 manuscripts are currently in preparation, both of which should be submitted by the fall of 2006.

We have over-expressed hsp90 in P69 cells and show no change in hsp90 levels (protein), no change in telomerase activity, and no change in growth rate. We suspect that these clones, all of which are selected in puromycin and remain drug resistant, show a feedback inhibition of endogenous hsp90, and we are currently looking at the RNA levels of exogenous and endogenous levels of hsp90.

We have ectopically expressed the p23 co-chaperone in the P69 nontumorigenic cells and show an increase in p23 without a concomitant increase in telomerase activity, suggesting that p23 is not limiting in these cells.

Over-expression of hsf-1 in P69 cells results in an increase in hsp90, hsp70, hsp27, and hsf-1, and importantly for our studies, telomerase activity is also increased after hsf-1 expression

We have also over-expressed hTERT in these nontumorigenic P69 cells, which showed a corresponding and consistent increase in telomerase activity without a change in chaperone protein levels, including hsp90, p23, and hsf-1, as well as hsp70.

We introduced the oncogenic form of ras into the P69 cells, which gave us a positive control for transformation and determine whether chaperones or telomerase were upregulated. Oncogenic ras (RasV12) expressing clones do not show a substantial increase in telomerase activity (variability is consistent with that observed for pBABEpuro clones) nor do they upregulate the hsp90, hsp70, or p23 chaperones.

We have tested all of the hsf-1, hTERT and ras clones for their growth rates and their ability to form colonies in soft agar, an in vitro assay to assess transforming ability of potentially tumorigenic cell lines. We find minimal differences in growth rate for all of the clones when compared to either uninfected P69 cells or vector (pBABEpuro) infected clones. We also observe a modest transforming ability with the vector controls and absolutely no colony formation with the hsf-1 clones in the soft agar assay. Curiously, we find a significant colony forming efficiency for the hTERT expressing P69 cells, yet oncogenic ras (RasV12) P69 cells produces significantly more colonies

One of our original hypotheses was that the increase in telomerase activity during progression was the result of elevated chaperone-mediated folding of the telomerase complex, rather than an increase in the expression of telomerase components (hTERT and hTR remain unchanged during transformation) (Akalin et al., 2001). Consequently, the expectation when ectopically expressing hTERT in the non-tumorigenic P69 cells was that there would be no increase in activity without an increase in assembly as chaperones are limiting, but interestingly, we observe a significant increase in activity after hTERT expression. Because endogenous hTERT is expressed at very low levels in most tumor-related cells, it is likely not a prominent target for the hsp90 chaperone complex, but when hTERT is suddenly over-expressed, it becomes more available for chaperone-mediated assembly. As such, we have subsequently shown that in hTERT over-expressing cells, more of the hsp90 and p23 chaperones are observed in the nucleus of the cells, suggesting more association of these chaperones with telomerase. We have determined this molecularly, showing that more hsp90 is associated with hTERT in the over-expressing cells, suggesting that telomerase is a more abundant chaperone target.

All of the above data was shown as figures last year. The initial data on hsp90, p23, hsf-1 over-expression and telomerase activity and lack of transformation is currently being put together as a paper by one of my former students, Dr. Keith O. Jensen, who did all of the work. He is also writing a manuscript on the hTERT over-expression for publication.

Objective #2: **Determine the cellular and molecular consequences of targeted inhibition of chaperones and/or telomerase using pharmacological and genetic approaches in tumorigenic prostate cancer cells.**

Despite several studies describing the effect of chaperone inhibition on telomerase activity, few studies have examined the long-term consequences of Hsp90 inhibition on telomere length using either pharmacological or genetic approaches. Because we observe an increase in chaperone expression and function in prostate cancer cell lines and primary prostate tumors (Akalin et al., 2001), our goal was to determine if chaperones, specifically hsp90, were targets for inhibition of telomerase activity and reversion of the tumorigenic phenotype to a less severe, non-tumorigenic state. All of the data on hsp90 inhibition was reported in last year's progress report, as well as in the attached manuscript (Compton et al., 2006).

We have some additional, albeit preliminary, data on the role of p23 in the NOS pathway, free radical production, and telomeres. We genetically inhibited p23 expression using siRNA,

showing a decline in p23 protein levels, no change in telomerase activity, and telomere shortening (Figure 3). It is important to note that while telomerase activity is not reduced in p23 siRNA cells, there may be a transient decline in activity that would not be observed after clonal selection, which takes 3 weeks. Similar results were obtained with the hsp90 siRNA cells.

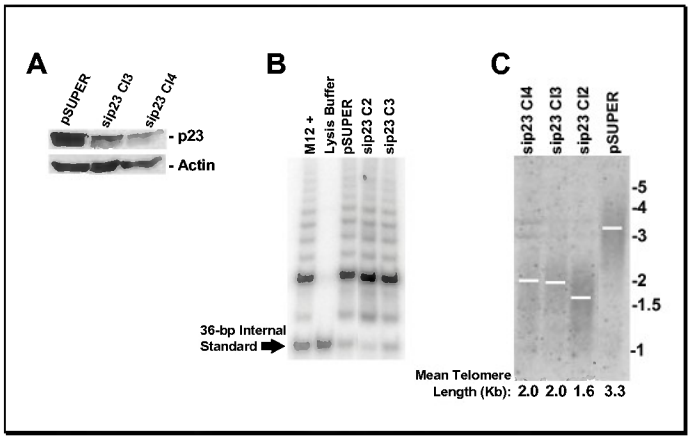

Figure 3. Characterization of P69 cells stably expressing siRNA to p23. **A.** Western analysis of p23 expression levels in representative clones of p23 with siRNA expressed shows significant reduction in p23 protein levels. **B.** Telomerase activity assay (TRAP – telomeric repeat amplification protocol) of clones with siRNA to p23, showing no change in telomerase activity. **C.** Telomere lengths of p23 siRNA clones are reduced in P69 cells.

Similar to hsp90 siRNA, we also tested cells with p23 siRNA for elevated levels of ROS (free radicals) as a means to explain the loss of telomere length. We observe a significant increase in ROS (Figure 4) and are currently looking into the role of p23 in the nitric oxide synthase pathway. We suspect that hsp90 requires p23 for NOS function, and a reduction in p23 renders hsp90 functionally inactive, causing a shift from nitric oxide production to the superoxide free radical being produced (Figure 5).

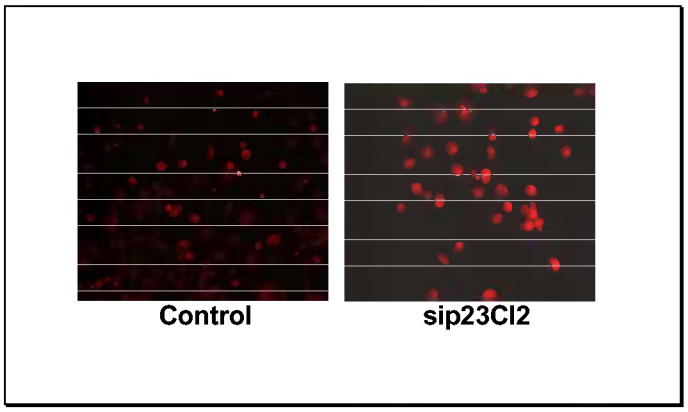

Figure 4. Elevated Reactive Oxygen Species (ROS) in P69 cells with reduction of p23 levels. Vector (pSUPER) controls show no substantial levels of free radicals after staining with dihydroethidium, while P69 cells with siRNA to p23 have a dramatic increase in ROS in nearly all cells assayed.

Our data supports a model in which Hsp90 regulates the production of nitric oxide ($^{\bullet}NO$) and superoxide (O^{2-}) through NOS, depending partially on the functional status of Hsp90 and its ability to uncouple NOS enzymatic activity. A consequence of Hsp90 deregulation is that NOS promotes the production of the O^{2-} free radical, causing extensive DNA damage and as our data shows, preferential targeting of telomeres (Figure 5).

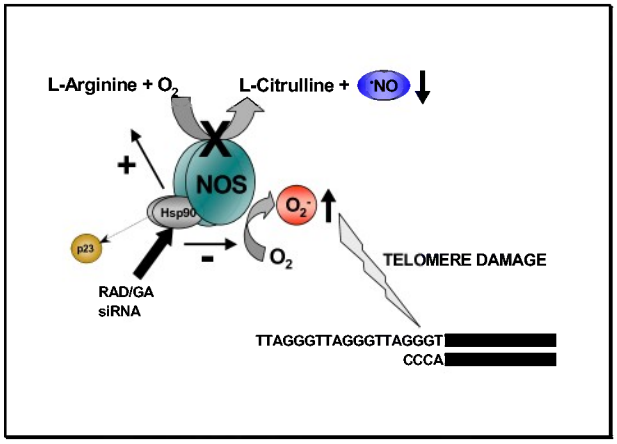

Figure 5. Model of Hsp90-Mediated Regulation of Nitric Oxide Synthase (NOS) and Free Radical-Induced Telomere Damage. NOS produces nitric oxide (˙NO) as a byproduct of the reaction in which L-Arginine and oxygen are converted to L-Citrulline. Hsp90 association with NOS promotes this conversion and suppresses the pathway by which NOS generates the superoxide (O^{2-}) free radicals. Inhibition of Hsp90 function pharmacologically (RAD) or genetically (siRNA) disrupts NOS conformation to inhibit ˙NO production and promote O^{2-}generation, which results in the accumulation of telomere damage (shortening), rendering the cell susceptible to apoptosis. L-NAME, by blocking both NOS pathways, protects against telomere damage and apoptosis (not depicted in model). Taken from Compton et al., 2006.

We have clearly shown that blocking hsp90 (or p23) function results in only a transient inhibition of telomerase activity and a significant telomere shortening in prostate tumor cells. Both pharmacologic and genetic inhibition of hsp90 results in an inability to maintain proper cellular homeostasis, causing a range of altered physiological responses including an imbalance of free radical production and telomere damage.

Key Research Accomplishments (over all 4 years of funding)

1-establishment of chaperone-expressing P69 cells that result in an increase in telomerase activity without transforming capabilities.

2-even though reduced telomerase activity was observed to be due to limiting chaperones, expression of hTERT results in increased telomerase activity in non-tumorigenic cells, suggesting that ectopic hTERT becomes a more prominent/available target for the hsp90 chaperone complex.

3-elevated levels of chaperones does not result in tumorigenic transformation, suggesting that the up-regulation of chaperones is a byproduct of prostate cancer progression.

4-blocking chaperone function with radicicol results in transient telomerase inhibition, telomere erosion, and eventually cell death in tumorigenic cell lines, indicating that targeting chaperones in tumorigenic prostate cancer cells may be an appropriate therapy.

5-genetic inhibition of hsp90 or p23 using siRNA results in telomere erosion as well.

6-telomere damage in the presence of telomerase is due to the deregulation of the nitric oxide synthase pathway, which is the result of hsp90 inhibition, and an increase in free radical production.

7-chaperones and/or telomeres may be a novel therapeutic targets for prostate cancer therapy.

Recommended Changes to the Proposed Work Based on Additional Findings

None requested.

Reportable Outcomes

Manuscripts

Compton,S.A., L.W.Elmore, K.Haydu, C.K.Jackson-Cook and **S.E.Holt**. 2006. Induction of NOS-Dependent Telomere Shortening after Functional Inhibition of Hsp90 in Human Tumor Cells. Molecular and Cellular Biology 26:1452-1462. (attached)

2 additional projects are near completion and will be written by the end of the summer of 2006.

We are also regenerating data on p23, NOS, and ROS for publication in a high profile journal.

Abstracts/Presentations

Gewirtz, D.A., D.Xu, C.Dumur, **S.E.Holt**, and L.W.Elmore. AACR Annual Meeting, April 2005.

Compton, S.A., L.W.Elmore, and **S.E.Holt.** Telomeres and Telomerase: Cold Spring Harbor Meeting, Cold Spring Harbor, NY. May 2005.

Jensen, K.O., L.W.Elmore, and **S.E.Holt.** Telomeres and Telomerase: Cold Spring Harbor Meeting, Cold Spring Harbor, NY. May 2005.

Elmore,L.W., X.Di., E.A.Gaskins, D.A.Gewirtz, and **S.E.Holt.** AACR Special Conference. La Jolla, CA. September 2005.

Invited Seminars

Holt,S.E. Dermatology Grand Rounds, MCV/VCU, Richmond, VA. November 2005.

Holt,S.E. University of Deleware, Newark, DE. October 2005.

Holt,S.E. Pathology Grand Rounds, MCV/VCU, Richmond, VA. September 2005.

Holt,S.E. Mount Desert Biological Laboratory, Salisbury Cove, ME. August 2005.

Holt,S.E. Massey Cancer Center, MCV/VCU, Richmond, VA. July 2005

Holt,S.E. Eppley Cancer Center, University of Nebraska at Omaha, Omaha, NE. June 2005.

Development of Cell Lines

As before, we have developed a number of cell lines for telomerase and chaperone over-expression in the non-tumorigenic prostate epithelial cell line, as well as cell lines with oncogenic ras over-expression. We have also made the corresponding tumorigenic cell lines knocking out hsp90, p23, and telomerase using the siRNA approach.

Funding Applied For

None during this period.

Conclusions

Our data conclusively shows that over-expression of telomerase on its own is not sufficient to elicit transformation nor does it allow for elevation of the chaperone proteins. Increased chaperone levels after hsf-1 expression has provided promising results related to stable expression and transformation, with minimal effect on tumorigenicity but significant effects on telomerase activity. We have also shown that over-expression of oncogenic ras results in tumorigenic transformation without significant effects on telomerase activity or chaperone levels. Together, all of our data suggests that transformation in prostate epithelial cells is very complex, and while chaperones may be important for the transformation process under some conditions, chaperone over-expression is not sufficient to drive transformation.

Our results for the pharmacologic and genetic inhibition of molecular chaperones and telomerase is not only interesting, but represents the first indirect method for a telomerase-independent, chaperone-mediated telomere shortening in a prostate cancer cell model system.

The role of ROS and the Nitric Oxide Synthase pathway after hsp90 and/or p23 inhibition has been defined and the data suggest that telomere shortening is induced by free radical generation after blocking hsp90/p23 function. Inhibition of chaperone function in tumorigenic prostate cells may represent a novel mode of prostate cancer therapy that would be useful for patients with more severe disease, which may provide a means of limiting recurrence or metastasis.

References (bold indicates PI authored papers)

Akalin,A., L.W.Elmore H.L.Forsythe, B.A.Amaker, E.D.McCollum, P.S.Nelson, J.L.Ware, and S.E.Holt. 2001. A novel mechanism for chaperone-mediated telomerase regulation during prostate cancer progression. Cancer Research. 61:4791-4796.

Bae VL, Jackson-Cook CK, Brothman AR, Maygarden SJ, and Ware JL. 1994. Tumorigenicity of SV40 T antigen immortalized human prostate epithelial cells: association with decreased epidermal growth factor receptor (EGFR) expression. Int J Cancer **58**:721-729.

Compton,S.A., L.W.Elmore, K.Haydu, C.K.Jackson-Cook and S.E.Holt. 2006. Induction of NOS-Dependent Telomere Shortening after Functional Inhibition of Hsp90 in Human Tumor Cells. Molecular and Cellular Biology 26:1452-1462.

Forsythe,H.L. J.L.Jarvis, J.W.Turner, L.W.Elmore, and S.E.Holt. 2001. Stable association of hsp90 and p23 with human telomerase. J Biol. Chem. 276:15571-15574.

CURRICULUM VITAE
February 2006

SHAWN EDAN HOLT, PH.D.

1. PERSONAL INFORMATION

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2. LICENSURE NONE

3. EDUCATION

1989-1994 Ph.D. in Genetics, Texas A&M University, College Station, TX
Dissertation: Sequence determinants for DNA binding specificity and transient DNA replication by the BPV-1 E1 protein
Major Professor: Van G. Wilson, Ph.D.

1985-1989 B.A. in Biology, The Colorado College, Colorado Springs, CO

4. MILITARY SERVICE NONE

5. POSTDOCTORAL TRAINING AND SPECIAL EXPERIENCE

1994-1998 Postdoctoral Fellow, Department of Cell Biology and Neuroscience, The University of Texas Southwestern Medical Center, Dallas, TX
Sponsors: Jerry W. Shay, Ph.D. and Woodring E. Wright, M.D., Ph.D.

1992-1993 Vice-President, The Graduate Student Government, Texas A&M University, College Station, TX

1991-1992 Chair, National Affairs, The Graduate Student Government, Texas A&M University, College Station, TX
Advisor: Harvey J. Tucker, Ph.D.

1989-1990 Graduate Teaching Assistant, Genetics Program, Texas A&M University, College Station, TX
Advisor: John R. Ellison, Ph.D.

6. APPOINTMENTS AND SIGNIFICANT EXPERIENCE

1998-2003 Assistant Professor, Department of Pathology, Department of Pharmacology and Toxicology, and Department of Human Genetics, Medical College of Virginia Campus at Virginia Commonwealth University, Richmond, VA

2003-present	Associate Professor, Department of Pathology, Department of Pharmacology and Toxicology, and Department of Human Genetics, Medical College of Virginia Campus at Virginia Commonwealth University, Richmond, VA
2003-present	Director, Graduate Education Program, Department of Pathology, Medical College of Virginia Campus at Virginia Commonwealth University, Richmond, VA
1998-present	Member, Massey Cancer Center, Medical College of Virginia Campus at Virginia Commonwealth University, Richmond, VA
2002-present	Member, Molecular Biology & Genetics Program, Medical College of Virginia Campus at Virginia Commonwealth University, Richmond, VA

7. PROFESSIONAL SOCIETIES

American Society for Virology (member since 1992)
American Society for Microbiology (member since 1995)
Society for Experimental Biology and Medicine (member since 1999)
American Association for Cancer Research (member since 1995)
Cell Stress Society International (member since 2002)
American Society of Biochemistry and Molecular Biology (member since 2002)
International Society for Stem Cell Research (member since 2004)
American Aging Association (member since 2004)

8. MEMBERSHIP IN COMMUNITY ORGANIZATIONS

1986-1988	Young Life Leader, Wasson High School, Colorado Springs, CO
1990-present	Alumni Admission Representative for The Colorado College, Colorado Springs, CO
1992	Science Fair Judge, A&M Consolidated High School, College Station, TX
1993	Volunteer, Special Olympics, College Station, TX
1997-1998	Science Fair Judge, Dallas, TX
2000-2003	Spring Run Athletic Association, Coach of soccer, baseball, basketball; Chesterfield County, VA
2001-2003	President, Hampton Park Board of Directors, Chesterfield, VA
2003-present	Chesterfield Little League, coach and manager (selected All Star manager in 2004)
2005-present	Richmond Kickers, assistant coach, Midlothian, VA

9. SPECIAL AWARDS, FELLOWSHIPS, & HONORS

9.1 Awards

2000-2003	The V Foundation Scholars Program, Cary, NC ($100,000, PI)
1994	Outstanding Presenter, Research Symposium, Texas A&M Health Science Center, College Station, TX
1994	Outstanding Graduate Student Government Member, Texas A&M University, College Station, TX
1988-1989	Dean's List, The Colorado College, Colorado Springs, CO
1988	Most Dedicated Football Player, The Colorado College, Colorado Springs, CO
1987	Rookie of the Year, Baseball, The Colorado College, Colorado Springs, CO
1985-1987	Outstanding College Students of America

9.2 Fellowship

1996-1998	NRSA Postdoctoral Research Fellowship, The National Institute on Aging

9.3 Grants

2004-2006	Department of Defense, Breast Cancer IDEA Award, ($441,000, PI)
2004-2006	Department of Defense, Breast Cancer Predoctoral Award for Kennon R. Poynter, ($87,000, Mentor)
2002-2005	Department of Defense, Prostate Cancer New Investigator Award, ($330,000, PI)
2002-2004	National Institute on Aging, R-21 (Co-investigator, ~$120,000 annually, PI: Colleen Jackson-Cook)
2002-2003	Mount Desert Island Biological Labs, New Investigator Award ($12,000, PI)
2001-2004	Department of Defense – Army, Breast Cancer IDEA Award (Co-PI) ($450,000 for 3 years, 10% effort; PI: David A. Gewirtz)
2001-2002	Department of Defense – Army, Breast Cancer Concept Award ($50,000, PI)
2001-2003	National Cancer Institute (Co-Invest., 10% effort) (P.I.: Alphonse E. Sirica, $190,125 direct annually)
2000-2002	The Mary Kay Charitable Foundation, Dallas, TX ($100,000, PI)
2000-2001	Commonwealth Health Research Board (Co-Invest.), Richmond, VA ($94,000 for 1 year, 15% effort)
1999-2000	Massey Cancer Center, Pilot Projects, MCV/VCU ($30,000 Co-PI)
1999-2000	Jeffress Memorial Trust, Richmond, VA ($30,000, PI)
1998-1999	Aid Grant IN-105, American Cancer Society, MCV/VCU, Richmond, VA ($15,000, PI)
1993	American Society for Virology Travel Grant
1993	Minigrant, Texas A&M University, College Station, TX

9.4 Invited Seminars

Holt,S.E. Department of Biology, Maggie Walker Governor's School, Richmond, VA. March 2006.
Holt,S.E. Pathology Grand Rounds, MCV/VCU, Richmond, VA. February 2006.
Holt,S.E. Dermatology Grand Rounds, MCV/VCU, Richmond, VA. November 2005.
Holt,S.E. Aquatic Animal Models of Human Disease, University of Georgia, Athens, GA. October 2005.
Holt,S.E. University of Deleware, Newark, DE. October 2005.
Holt,S.E. Pathology Grand Rounds, MCV/VCU, Richmond, VA. September 2005.
Holt,S.E. Mount Desert Biological Laboratory, Salisbury Cove, ME. August 2005.
Holt,S.E. Massey Cancer Center, MCV/VCU, Richmond, VA. July 2005
Holt,S.E. Eppley Cancer Center, University of Nebraska at Omaha, Omaha, NE. June 2005.
Holt,S.E. Keynote Speaker, The Colorado College Biology Day, Colorado Springs, CO. April 2005.
Holt,S.E. Department of Biology, Maggie Walker Governor's School, Richmond, VA. March 2005.
Holt,S.E. Keynote Speaker, Pugwash Conference, Richmond, VA. March 2005.
Holt,S.E. MD/PhD Program, MCV/VCU, Richmond, VA. December 2004.
Holt,S.E. and S.A.Compton. AACR: Telomeres/telomerase in cancer. San Francisco, CA. November 2004.
Holt,S.E. Mount Desert Island Stem Cell Symposium, MDIBL, Salisbury Cove, ME. August 2004.
Holt,S.E. Department of Biology, Maggie Walker Governor's School, Richmond, VA. March 2004.
Holt,S.E. Department of Physiology, MCV/VCU, Richmond, VA. February 2004.
Holt,S.E. Department of Biological Sciences, Texas Tech University, Lubbock, TX. October 2003.
Holt,S.E. Department of Human Genetics, MCV/VCU, Richmond, VA. September 2003.
Holt,S.E. Mini-Medical School, Science Museum of Virginia, Richmond, VA. March 2003.
Holt,S.E. Department of Neurooncology, MCV/VCU, Richmond, VA. March 2003.
Holt,S.E. Department of Biology, Maggie Walker Governor's School, Richmond, VA. March 2003.
Holt,S.E. Bridges Program, Virginia State University, Petersburg, VA. November 2002.

Holt,S.E. Molecular Biology and Genetics Program, MCV/VCU, Richmond, VA. October 2002.
Holt,S.E. Department of Pharmacology/Toxicology, MCV/VCU, Richmond, VA October 2002.
Holt,S.E. Department of Chemistry, MCV/VCU, Richmond, VA. September 2002.
Holt,S.E. Stem Cells on Land and at Sea. Mount Desert Island Biological Laboratory and Jackson Laboratory, Bar Harbor, ME. August 2002.
Holt,S.E. Department of Medicine, University of Colorado Health Science Center, Denver, CO. June 2002.
Holt,S.E. Department of Medical Microbiology and Immunology, Texas A&M Health Science Center, College Station, TX. April 2002.
Holt,S.E. Department of Biochemistry and Genetics, University of Texas Health Center at Tyler, Tyler, TX. April 2002.
Holt,S.E. Department of Biology, Maggie Walker Governor's School, Richmond, VA. March 2002.
Holt,S.E. Department of Physiology & Neurobiology, University of Connecticut, Storrs, CT. February 2002.
Holt, S.E. Massey Cancer Center, MCV/VCU. February 2002.
Holt,S.E. Department of Pharmacology/Toxicology, Yale University, New Haven, CT. January 2002.
Holt,S.E. Department of Pathology, MCV/VCU. September 2001.
Holt,S.E. Department of Human Genetics, MCV/VCU. September 2001.
Holt,S.E. Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, NE. June 2001.
Holt,S.E. Generation of New Marine Cell Lines and Transgenic Species. Mount Desert Island Biological Laboratory, Salisbury Cove, ME. May 2001.
Holt,S.E. American Type Cell Culture, Manassas, VA. May 2001.
Holt,S.E. Telomeres & Telomerase. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. March 2001.
Holt,S.E. Governor's School, Richmond, VA. March 2001.
Holt,S.E. Keynote Speaker, Pugwash Conference, Richmond, VA. March 2001.
Holt,S.E. National Institute for Allergy and Infectious Disease, National Institutes of Health. October 2000.
Holt,S.E. National Heart, Lung, and Blood Institute, National Institutes of Health. October 2000.
Holt,S.E. The Geron Symposium #3: Telomeres and Telomerase in Aging and Cancer. San Francisco, CA. June 2000.
Holt,S.E. The Sidney Kimmel Cancer Center, La Jolla, CA. June 2000.
Holt,S.E. Emporia Community Hospital, Emporia, VA. April 2000.
Holt,S.E. Department of Physiology, MCV/VCU. Richmond, VA. February 2000.
Holt,S.E. Neuro-Oncology Forum, MCV/VCU. Richmond, VA. January 2000.
Holt,S.E. Southside Regional Medical Center, Petersburg, VA. January 2000.
Holt,S.E. Massey Cancer Center, MCV/VCU. Richmond, VA. December 1999.
Holt,S.E. Department of Biology, University of Richmond. Richmond, VA. November 1999.
Holt,S.E. Department of Pathology, University of Colorado Health Science Center. Denver, CO. October 1999.
Holt,S.E. Department of Biology, The Colorado College. Colorado Springs, CO. October 1999.
Holt,S.E. Intergen Corporation. Gaithersburg, MD. May 1999.
Holt,S.E. Department of Biological Sciences, Texas Tech University. Lubbock, TX. February 1999.
Holt,S.E. Pathology Grand Rounds, MCV/VCU. Richmond, VA. February 1999.
Holt,S.E. Massey Cancer Center, MCV/VCU. Richmond, VA. October 1998.
Holt,S.E. Department of Human Genetics, MCV/VCU. Richmond, VA. September 1998
Holt,S.E. Telomerase and Telomere Dynamics in Cancer and Aging. Kapalua, Maui, HI. August 1998.
Holt,S.E. Sapporo Cancer Seminar. Hokaido University, Sapporo, Japan. July, 1998.
Holt,S.E. Telomeres and Telomerase: Immortality, Cancer and Aging. San Francisco, CA. June 1998.
Holt,S.E. Department of Biology, The Colorado College. Colorado Springs, CO, October 1997.

Holt,S.E. Geron Telomerase and Cancer Symposium. Kona, Hawaii, August 1996.

10. Major Committees

10.1 University Committees

1992-1994	Graduate Appeals Panel, Texas A&M University, College Station, TX
1993	Chair, Hurricane Andrew Educational Relief Drive, Texas A&M University, College Station, TX
1993	AIDS Awareness Committee, Texas A&M University, College Station, TX
1994	Chair, Third Annual Graduate Research Symposium, Texas A&M Health Science Center, College Station, TX
1998-present	Student advisory committees (total of 35 overall)
1999-present	Chair, Pathology Grand Rounds Committee, VCU
1999-present	Research Strategic Planning, Department of Pathology, VCU
2000-present	Member, Functional Genomics Program, VCU
2001-2003	Member, Pathology Search Committee, VCU
2002-present	Member, Molecular Biology and Genetics Program, VCU
2002-present	Member, Pathology Research Planning Group, VCU
2003-present	Director, Pathology Graduate Education, VCU
2003-present	Member, MCV Graduate Committee, VCU
2004-present	Member, Human Genetics Curriculum Committee, VCU
2004-present	Member, Medical Genetics and Pathology Fellowship Program, VCU

10.2 Professional Committees

2001-present	Editorial Board, Clinical Cancer Research (Editor: John Mendelson)
2001-present	Member, Medical Genetics Review Panel, Department of Defense Breast Cancer Research Program, Washington DC
2001-present	British Cancer Research Fund, Ad Hoc Reviewer, London
2002-2005	National Science Foundation, Ad Hoc Reviewer, Washington DC
2002-present	Editorial Board, Cancer Biology and Therapy (Editor: Wafik El-Diery)
2003	Phillip Morris External Research Program, Ad Hoc Reviewer, Richmond
2003	Austrian Science Fund (FWF), Ad Hoc Reviewer, Austria
2004-present	National Scientific Advisory Council, The American Federation for Aging Research, External Grant Program, Scientist Reviewer
2003-present	Reviewer, Concept Award Program, Department of Defense Breast Cancer Research Program, Washington DC
2003-present	Member, Molecular Biology and Genetics Review Panel, Department of Defense Prostate Cancer Research Program, Washington DC
2004-present	Editorial Board, Society for Experimental Biology and Medicine
2004-present	Member, Molecular Biology and Genetics #2 Review Panel, Department of Defense Breast Cancer Research Program, Washington DC
2005-present	Member, Pathogenesis Review Committee, California Breast Cancer Research Program, San Francisco, CA
2005-present	Member, Eukaryotic Genetics Review Panel, National Science Foundation, Washington DC

11. Other Scholarly Activity

11.1 Students (past and present)

Ali Akalin, M.D., Ph.D.	Ph.D. in Pathology, 2000 – "The Role of Telomerase in Prostate Cancer Progression" [dissertation] (Major Professor)
Graham Strub	M.A. in Human Genetics, 2003 – "The Effects of Heavy Metal Salts on Human Fibroblast Growth, HSP Expression, Telomerase Activity, and Telomere Erosion" [thesis] (Major Professor)
Sarah Compton	Ph.D. in Pharmacology/Toxicology (Major Professor), 2004 – "Pharmacological and Genetic Analysis of Chaperone Proteins Hsp90 and p23 in Telomerase and Telomere Biology" [dissertation] (Major Professor)
Matthew Rapp	M.A. in Human Genetics, 2005 – "Optimization of the Telomerase Assay and Characterization of the Telomerase Complex" [thesis] (Major Professor)
Keith Jensen	1999-present, Ph.D. program in Human Genetics (Major Professor), anticipated graduation, May of 2006
Jeremy Aisenberg	2003-present, M.A. program in Human Genetics (Major Professor), anticipated graduation, May of 2006
Kennon Poynter	2002-present, Ph.D. program in Human Genetics (Major Professor), anticipated graduation, December of 2006
Binh Nguyen	2002-present, M.D./Ph.D. program in Pathology (Major Professor), anticipated graduation from PhD portion, May of 2007
Amy Depcrynski	2004-present, Ph.D. program in Human Genetics (Major Professor), anticipated graduation, August 2008
Malissa Liu	2004-present, Ph.D. program in Human Genetics (Major Professor), anticipated graduation, August 2008
Patrick Sachs	2004-present, Ph.D. program in Human Genetics (Major Professor), anticipated graduation, August 2009
Medical Students	Vince Lawson, John Turner, Eric McCollum, Shaveta Vinayak, Robert Ferguson, Quintesia Grant (Natl Medical Fellowship funded student), Neil Haycocks, Jon Hlivko, Elizabeth Gaskins, Bret Adams
Undergraduates	Jennie Jarvis (HHMI funded student), Jeannie Siri, Anne Graves, Sara Thayer, Byrd Davenport, Taylor Bright, Melissa Landon, Patrick Sachs, Lisa Richter, Edward Henderson, Eda Kapinova
High School	Theodora Daniel, Calvin Dawson, Taylor Bright, Matthew Lankowski, Suravi Sircar, Katelyn Bowman, Sohini Sircar

11.2 Fellows

Heidi L. Forsythe, D.P.M.	1998-2002 (stay at home mom in Boulder, CO)
Chunxiao Zhou, M.D., Ph.D.	1999-2000 (postdoctoral fellow at UNC Chapel Hill, NC)
Ali Akalin, M.D., Ph.D.	2000-2001 (Pathology resident at UCHSC Denver, CO)
Patricia A. McChesney, Ph.D.	2001-2004 (junior faculty at UVa Charlottesville, VA)
Sarah A. Compton, Ph.D.	2004-2004 (postdoctoral fellow at UNC Chapel Hill, NC)

11.3 Teaching Assignments

HGEN 614 – Biochemical Genetics (3 lectures with organizational assistance)
HGEN 511 – Cytogenetics (2 lectures)
BIOC 605 – Molecular Biology (2 lectures)
PMTX 632 – Cancer Genetics (2 lectures)
PATH 570 – Experimental Approaches to Tumor Biology (6 lectures)
Lectures in various courses on aging and cancer

12. BIBLIOGRAPHY

12.1 Papers Published

1-Holt,S.E., G.Schuller and V.G.Wilson. 1994. DNA binding specificity of the Bovine Papillomavirus E1 protein is determined by sequences contained with an 18 bp inverted repeat element at the origin of replication. Journal of Virology **68**: 1094-1102.

2-Schuller,G., **S.E.Holt**, J.Hsu and V.G.Wilson. 1994. The Bovine Papillomavirus type 1 genome contains multiple loci of static DNA bending, but bends are absent from the functional origin of replication. Virus Research **31**: 203-217.

3-Holt,S.E. and V.G.Wilson. 1995. Mutational analysis of the 18 bp inverted repeat element at the Bovine Papillomavirus origin of replication: Identification of critical sequences for E1 binding and in vivo replication. Journal of Virology **69**: 6525-6532.

4-Holt,S.E., L.S.Gollahon, T.Willingham, M.S.Barbosa, and J.W.Shay. 1996. p53 levels in human mammary epithelial cells expressing wild-type and mutant human papillomavirus type 16 (HPV-16) E6 proteins: relationship to reactivation of telomerase and immortalization. International Journal of Oncology **8**: 263-270.

5-Schroeder,M., S.Miller, V.Srivastava, E.Merriam-Crouch, **S.Holt**, V.Wilson, and D.Busbee. 1996. DNA polymerase α accessory protein (αAP) enhances both DNA binding and activity of DNA polymerase α isolated from aged donors. Mutation Research **316**: 237-248.

6-Holt,S.E., W.E.Wright, and J.W.Shay. 1996. Regulation of telomerase activity in immortal cell lines. Molecular and Cellular Biology **16**: 2932-2939.

7-Holt,S.E., J.W.Shay, and W.E.Wright. 1996. Refining the telomere-telomerase hypothesis of aging and cancer. Nature Biotechnology **14**: 836-839.

8-Holt,S.E., J.C.Norton, W.E.Wright, and J.W.Shay. 1996. Comparison of the telomeric repeat amplification protocol (TRAP) to the new TRAP-eze telomerase detection kit. Methods in Cell Science **18**: 237-248.

9-Holt,S.E., W.E.Wright, and J.W.Shay. 1997. Multiple pathways for the regulation of telomerase activity. European Journal of Cancer **33**: 761-766.

10-Holt,S.E., D.L.Aisner, J.W.Shay, and W.E.Wright. 1997. Lack of cell cycle regulation of telomerase activity in human cells. Proceedings of the National Academy of Sciences, USA, **94**: 10687-10692.

11-Weinrich,S.L., R.Pruzan, L.Ma, M.Ouellette, V.M.Tesmer, **S.E.Holt**, A.G.Bodnar, S.Lichtsteiner, N.W.Kim, J.B.Trager, R.D.Taylor, R.Carlos, W.H.Andrews, W.E.Wright, J.W.Shay, C.B.Harley, and G.B.Morin. 1997. Reconstitution of human telomerase with the template RNA hTR and the catalytic protein subunit hTRT. Nature Genetics **17**: 498-502.

12-Bodnar,A.G., M.Ouellette, M.Frolkis, **S.E.Holt,** C-P.Chiu, G.B.Morin, C.B.Harley, J.W.Shay, S.Lichtsteiner and W.E.Wright. 1998. Extension of life-span by introduction of telomerase in normal human cells. Science **279**: 349-352.

13-Norton,J.C., **S.E.Holt**, W.E.Wright, and J.W.Shay. 1998. Enhanced detection of telomerase activity. DNA and Cell Biology **17**: 217-219.

14-Morales CP, **S.E.Holt**, M.Ouellette, K.Kaur, Y.Yan, K.S.Wilson, M.A.White, W.E.Wright, and Shay JW. 1999. Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. Nature Genetics **21**:55-58.

15-Holt,S.E., D.L.Aisner, J.Baur, V.M.Tesmer, M.Dy, M.Ouellette, D.O.Toft, J.B.Trager, G.B.Morin, W.E.Wright, J.W.Shay, and M.A.White. 1999. Functional requirement of p23 and hsp90 in telomerase complexes. Genes and Development **13**:817-826.

16-Holt,S.E. and J.W.Shay. 1999. The role of telomerase in cellular proliferation and cancer. Journal of Cellular Physiology **180**:10-18.

17-Holt,S.E., V.V.Glinsky, A.B.Ivanova, and G.V.Glinsky. 1999. Resistance to apoptosis in human cells conferred by telomerase function and telomere length. Molecular Carcinogenesis **25**:241-248.

18-Tesmer,V.M., L.P.Ford, **S.E.Holt**, B.C.Frank, X.Yi, D.L.Aisner, M.Ouellette, J.W.Shay, and W.E.Wright. 1999. Two inactive fragments of the integral RNA cooperate to assemble active telomerase with the human protein catalytic subunit (hTERT) in vitro. Molecular and Cellular Biology **19**:6207-6216.

19-Savre-Train,I., L.S.Gollahon, and **S.E.Holt**. 2000. Clonal heterogeneity in telomerase activity and telomere length in tumor-derived cell lines. Proceedings of the Society for Experimental Biology & Medicine **223**:379-388.

20-Ouellette,M., M.Liao, B.Shea-Herbert, M.Johnson, **S.E.Holt**, H.S.Liss, J.W.Shay, and W.E.Wright. 2000. Subsenescent Telomere Lengths in Fibroblasts Immortalized by Limiting Amounts of Telomerase. Journal of Biological Chemistry **275**:10072-10076.

21-Elmore,L.W. and **S.E.Holt**. 2000. Telomerase and telomere stability: A new class of tumor suppressor? Molecular Carcinogenesis **28**:1-4.

22-Gollahon,L.S. and **S.E.Holt**. 2000. Alternative methods of extracting telomerase activity from human tumor and tissue samples. Cancer Letters **159**:141-149.

23-Forsythe,H.L. J.L.Jarvis, J.W.Turner, L.W.Elmore, and **S.E.Holt.** 2001. Stable association of hsp90 and p23 with human telomerase. J Biol. Chem. **276**:15571-15574.

24-Akalin,A., L.W.Elmore H.L.Forsythe, B.A.Amaker, E.D.McCollum, P.S.Nelson, J.L.Ware, and **S.E.Holt**. 2001. A novel mechanism for chaperone-mediated telomerase regulation during prostate cancer progression. Cancer Research. **61**:4791-4796.

25-Holt,S.E., E.J.Brown, and A.Zipursky. 2002. A study of telomerase levels in the leukemic cells of patients with the megakaryoblastic leukemias of Down syndrome. J Ped Hemat/Oncol. **24**:14-17.

26-Holt,S.E. 2002. Is Telomerase the Cure-All and End-All? Drug Discovery Today **7**:288.

27-Forsythe,H.L., K.O.Jensen, L.W.Elmore, and **S.E.Holt.** 2002. Ectopic expression of telomerase in human cells provides a growth advantage, extends proliferative life span, and rescues near senescent cells. International Journal of Oncology **20**:1137-1143.

28-McKinstry,R., L.Qiao, A.Yacoub, Y.Dai, R.Decker, **S.Holt**, M.P.Hagan, S.Grant, and P.Dent. 2002. Inhibitors of MEK1/2 Interact with UCN-01 to Induce Apoptosis and Reduce Colony Formation in Mammary and Prostate Carcinoma Cells. Cancer Biology and Therapy **1**:243-253.

29-Harvey,S.A., K.O.Jensen, L.W.Elmore, and **S.E.Holt**. 2002. Pharmacological approaches to defining the role of chaperones in aging and prostate cancer progression. Cell Stress and Chaperones **7**:230-234.

30-Elmore,L.W., H.L.Forsythe, A.Ferreira-Gonzalez, C.T.Garrett, G.M.Clark, and **S.E.Holt.** 2002. Real-Time Quantitative Analysis of Telomerase Activity in Breast Tumor Specimens using a Highly Specific and Sensitive Fluorescent-based Assay. Diagnostic Molecular Pathology **11**: 177-185.

31-Elmore,L.W., K.C.Turner, L.S.Gollahon, M.R.Landon, C.K.Jackson-Cook, and **S.E.Holt**. 2002. Telomerase protects cancer-prone cells from chromosomal instability and spontaneous immortalization. Cancer Biology and Therapy **1**:391-397.

32-Elmore,L.W., C.W. Rehder, M.Di, P.A.McChesney, C.K.Jackson-Cook, D.A.Gewirtz, and **S.E.Holt**. 2002. Adriamycin-induced replicative senescence in tumor cells requires functional p53 and telomere dysfunction. J. Biol. Chem. **277**:35509-35515.

33-McChesney,P.A., L.W.Elmore, and **S.E.Holt**. 2002. Aging and oxidative damage: are telomeres the target? Comments on Theoretical Biology **7**:295-313.

34-Nguyen,B., L.W.Elmore, **S.E.Holt**. 2003. Telomerase as a target for cancer immunotherapy. Cancer Biol Ther. **2**:131-6.

35-Elmore,L.W., P.A.McChesney, M.Lankowski, and **S.E.Holt**. 2003. Identification of high levels of telomerase in marine animals. The Bulletin, Mount Desert Island Biological Laboratory **42**:40-42.

36-Wise,S.S., L.W.Elmore, **S.E.Holt**, J.E.Little, P.G.Antonucci, B.H.Bryant, and J.P.Wise, Sr. 2004. Telomerase-Mediated Lifespan Extension Of Human Bronchial Cells Does Not Affect Hexavalent Chromium-Induced Cytotoxicity or Genotoxicity. Molecular and Cellular Biochemistry **255**:103-111.

37-Ossum,C.G., E.K.Hoffman, M.M.Vijayan, **S.E.Holt**, and N.C.Bols. 2004. Isolation and characterization of a novel fibroblast-like cell line from the rainbow trout (Oncohynchus mykiss) and a study of p38MAPK activation and induction of HSP70 in response to chemically-induced ischemia. Journal of Fish Biology **64**:1103-1116.

38-McChesney,P.A., K.C.Turner, L.W.Elmore, C.K.Jackson-Cook, and **S.E.Holt**. 2004. Telomerase Resets the Homeostatic Telomere Length and Prevents Telomere Dysfunction in Immortalized Human Cells. DNA and Cell Biology **23**:293-300.

39-Nguyen,B., L.W.Elmore, and **S.E.Holt**. 2004. Telomere Maintenance: at the Crossroads of Mismatch Repair? Cancer Biology and Therapy **3**:293-295.

40-Elmore,L.W., A.Parton, D.A.Barnes, and **S.E.Holt**. 2004. A novel role for telomerase in fish. The Bulletin, Mount Desert Island Biological Laboratory **43**:65-67.

41-Leach,N.T., C.Rehder C, K.Jensen K, **S.Holt**, and C.Jackson-Cook. 2004. Human chromosomes with shorter telomeres and large heterochromatin regions have a higher frequency of acquired somatic cell aneuploidy. Mech Ageing Dev. **125**:563-73.

42-McChesney,P.A., L.W.Elmore, and **S.E.Holt**. 2005. Vertebrate Marine Species as Model Systems for Studying Telomeres and Telomerase. Zebrafish, **1**:349-355.

43-Elmore,L.W., X.Di, Y-M.Di, **S.E.Holt**, and D.A.Gewirtz. 2005. Evasion of chemotherapy-induced senescence in breast cancer cell: implications for treatment response. Clin. Cancer Res. **11**:2637-43.

44-Jones,K.R., L.W.Elmore, L.Povirk, **S.E.Holt**, and D.A.Gewirtz. 2005. Reciprocal regulation of senescence and apoptosis in response to radiation in the breast tumor cell. International Journal of Radiation Biology, **81**:445-458.

45-Lai,G-H., Z.Zhang, X-N.Shen, D.J.Ward, J.L.DeWitt, **S.E.Holt**, R.A.Rozich, D.C.Hixson, and A.E.Sirica. 2005. *erbB2/neu* transformed rat cholangiocytes recapitulate key cellular and molecular features of human bile duct cancer. Gastroenterology, **129**:2047-2057.

46-Compton,S.A., L.W.Elmore, K.Haydu, C.K.Jackson-Cook and **S.E.Holt**. 2006. Induction of NOS-Dependent Telomere Shortening after Functional Inhibition of Hsp90 in Human Tumor Cells. Molecular and Cellular Biology **26**:1452-1462..

47-Elmore,L.W., E.Kapinovai, S.Sircar, S.Sircar, and **S.E.Holt**. 2006. Characterization of Telomerase Function in Cell Lines from Japanese Medaka. The Bulletin, MDIBL, in press.

48-Poynter,KP., L.W.Elmore, and **S.E.Holt**. 2006. Telomeres and telomerase in aging and cancer: lessons learned from experimental model systems. Drug Discovery Today, in press.

49-Rehder,R.C., P.McChesney, **S.E.Holt**, L.A.Corey, B.P.Riley, and C.K.Jackson-Cook. 2006. Chromosome-specific telomeres: Their lengths are inversely correlated with acquired aneuploidy levels and are influenced by heritable genetic factors. Submitted, Human Molecular Genetics.

50-Bryson, S.P., E.M. Joyce, D.J. Martell, L.E.J. Lee, **S.E. Holt**, K. Fujiki, B. Dixon and N.C. Bols. 2006. Development of a cell line, HEW, from embryos of haddock (*Melanogrammus aeglefinius*) and defining its capacity to tolerate environmental extremes. Submitted, Marine Biotechnology.

51-Elmore,L.W., McChesney,P.A., M.Landon, M.Lankowski, S.Sircar, S.Sircar, and **S.E.Holt**. 2006. Functional Conservation of Telomerase: from Marine Animals to Humans. Submitted to J.Biol.Chem.

52-Elmore,L.W., X.Di, E.Gaskins, D.A.Gewirtz, and **S.E.Holt**. 2006 Induction of a Breast Tumor Cell-Specific Senescence Bystander Effect. In preparation.

53-Forsythe,R., L.W.Elmore, H.L.Forsythe, S.Nasim, and **S.E.Holt**. 2006. Association of chaperones and telomerase in prostate tumor samples. In preparation.

12.2 Abstracts and Presentations

1-Holt,S.E., X.Leng and J.H.Ludes-Meyers. Sam Houston State University, Huntsville, TX. April 1992.

2-Holt,S.E. and V.G.Wilson. Second Annual Graduate Research Symposium and Poster Competition. Texas A&M Health Science Center, College Station, TX. September 1992.

3-Holt,S.E. and V.G.Wilson. Lost Pines Molecular Biology Conference, Smithville, TX. October 1992

4-Wilson,V.G., J.H.Ludes-Meyers, **S.E.Holt** and X.Leng. McGill University Conference on Regulation of Eukaryotic DNA Replication, Montreal, Canada. October 1992.

5-Holt,S.E. and V.G.Wilson. American Society for Virology, University of California, Davis, CA. July 1993.

6-Holt,S.E., G.Schuller and V.G.Wilson. XII International Papillomavirus Conference, Baltimore, MD. September 1993.

7-Holt,S.E., G.Schuller and V.G.Wilson. The Texas Branch of the American Society for Microbiology, Austin, TX. November 1993.

8-Holt,S.E., G.Schuller and V.G.Wilson. Third Annual Graduate Research Symposium and Poster Competition. Texas A&M Health Science Center, College Station, TX. March 1994.

9-Holt,S.E., G.Schuller and V.G.Wilson. Texas A&M University and Baylor College of Medicine Graduate Research Symposium, Texas A&M University, College Station, TX. April 1994.

10-Holt,S.E. Department of Medical Microbiology and Immunology, Texas A&M Health Science Center, College Station. August 1994.

11-Holt,S.E., G.Schuller and V.G.Wilson. Lost Pines Molecular Biology Conference, Smithville, TX. October 1994.

12-Holt,S.E., L.S.Gollahon, T.Willingham, M.S.Barbosa, and J.W.Shay. American Society for Virology, Austin, TX. July 1995.

13-Holt,S.E., P.H.Pompa, M.A.Piatyszek, L.S.Gollahon, W.E.Wright, and J.W.Shay. Workshop on Neoplastic Transformation in Cultured Human Cell Systems in Cultures: Mechanisms of Carcinogenesis, Chicago, IL. September 1995.

14-Holt,S.E., P.H.Pompa, M.A.Piatyszek, L.S.Gollahon, W.E.Wright, and J.W.Shay. American Association for Cancer Research. Washington D.C., April 1996.

15-Norton,J.C, L.S.Gollahon, **S.E.Holt**, W.E.Wright, and J.W.Shay. Lost Pines Molecular Biology Conference. Bastrop, Texas, October 1996.

16-Holt,S.E., L.S.Gollahon, I.Savre-Train, W.E.Wright, and J.W.Shay. Lost Pines Molecular Biology Conference. Bastrop, Texas, October 1996.

17-Holt,S.E., L.S.Gollahon, I.Savre-Train, W.E.Wright, and J.W.Shay. American Association for Cancer Research. San Diego, CA, April 1997.

18-Norton,J.C, L.S.Gollahon, **S.E.Holt**, W.E.Wright, and J.W.Shay. American Association for Cancer Research. San Diego, CA, April 1997.

19-Glinsky,G.V., V.V.Glinsky, **S.E.Holt**, and J.W.Shay. American Association for Cancer Research. New Orleans, LA, April 1998.

20-Morin,G.B., S.L.Weinrich, R.Pruzan, L.Ma, M.Ouellette, V.M.Tesmer, **S.E.Holt**, A.G.Bodnar, S.Lichtsteiner, N.W.Kim, J.B.Trager, R.D.Taylor, R.Carlos, W.H.Andrews, W.E.Wright, J.W.Shay, C.B.Harley. American Association for Cancer Research. New Orleans, LA, April 1998.

21-Holt,S.E. American Association for Cancer Research. Philadelphia, PA. April 1999.

22-Akalin,A., H.S.Liss, A.Ferreria-Gonzalez, C.T.Garrett, J.L.Ware, and **S.E.Holt.** Association for Molecular Pathology. St. Louis, MO. November 1999.

23-Elmore,L.W., A.Akalin, L.S.Gollahon, G.M.Clark, M.M.Grimes, R.T.Burks, A.Ferreira-Gonzalez, C.T.Garrett, and **S.E.Holt**. Molecular Biology of Breast Cancer Conference. Lillihammer, Norway. March 2000.

24-Forsythe,H.L., L.W.Elmore, K.O.Jensen, and **S.E.Holt.** Geron symposium #3: Telomeres and telomerase in aging and cancer. San Francisco, CA. June 2000

25-Elmore,L.W., M.R.Landon, A.Akalin, L.S.Gollahon, H.L.Forsythe, K.C.Turner, C.K.Jackson-Cook, **S.E.Holt.** Geron symposium #3: Telomeres and telomerase in aging and cancer. San Francisco, CA. June 2000.

26-Holt,S.E. Geron symposium #3: Telomeres and telomerase in aging and cancer. San Francisco, CA. June 2000.

27-Wise,S.S., L.W.Elmore, P.G.Antonucci, **S.E.Holt**, B.H.Bryant, and J.P.Wise. Northeast Society of Toxicology, Groton, CT. October 2000.

28-Wise,S.S., L.W.Elmore, P.G.Antonucci, **S.E.Holt**, B.H.Bryant, and J.P.Wise. American College of Toxicology, San Diego, CA. November 2000.

29-Jensen,K.O., A.Akalin, H.L.Forsythe, L.W.Elmore, and **S.E.Holt**. Telomeres and Telomerase, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. March 2001.

30-Forsythe,H.L., J.L.Jarvis, J.W.Turner, L.W.Elmore, and **S.E.Holt**. Telomeres and Telomerase, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. March 2001.

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Existing/Pending Support

Active

P.I.: Kennon R. Poynter
Mentor: Shawn E. Holt, Ph.D.
Title: Mechanisms of telomerase inhibition using small inhibitory RNAs and induction of breast tumor cell sensitization
Agency: Department of Defense Breast Cancer program
Duration: 4/1/04-3/31/07

P.I.: Lawrence F. Povirk, Ph.D. (Co-I, 5% effort, Shawn E. Holt, Ph.D.)
Title: Tyrosyl-DNA phosphodiesterase and oxidative DNA damage
Agency: NIH
Duration: 6/1/04-5/31/09

P.I.: Shawn E. Holt, Ph.D.
Title: Defining the regulation of telomerase through identification of mammary-specific telomerase interacting proteins
Agency: Department of Defense Breast Cancer program (grant # **W81XWH-04-0551)**
Duration: 6/1/04-5/31/07

P.I.: Lynne W. Elmore, Ph.D.
Mentors: Shawn E. Holt, Ph.D. and David A. Gewirtz, Ph.D.
Title: Genomic instability and senescence in breast cancer
Agency: NIH (K-08, Temin Award)
Duration: 9/1/05-8/31/10

P.I.: Colleen K. Jackson-Cook, Ph.D. (Co-I, 10% effort, Shawn E. Holt, Ph.D.)
Title: Aging and Genomic Changes: Role of Environment/Genetics
Agency: NIH/NIEHS
Duration: 12/1/05-11/30/10

Pending

P.I.: Shawn E. Holt, Ph.D.
Title: Mechanisms of Drug-Induced Telomere Dysfunction
Agency: NIH
Duration: 9/1/06-8/31/11

Completed

P.I.: David A. Gewirtz, Ph.D. (Shawn E. Holt, Ph.D., Co-PI, 10% effort)
Title: Reciprocal regulation of senescence/apoptosis in response to adriamycin in the breast tumor cell
Agency: Department of Defense
Duration: 7/1/01-6/30/05

P.I.:	Shawn E. Holt, Ph.D.
Title:	Mechanisms of prostate cancer transformation
Agency:	Department of Defense Prostate Cancer program
Duration:	12/18/01-12/17/05

P.I.:	Shawn E. Holt, Ph.D. (post-doctoral fellowship award)
Title:	Utilization of chaperone inhibition in combination with conventional cancer therapy to prevent breast tumor cell recovery
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Induction of Nitric Oxide Synthase-Dependent Telomere Shortening after Functional Inhibition of Hsp90 in Human Tumor Cells

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In most cancer cells, the lengths of telomeres, the functional DNA-protein complexes located at chromosome ends, are maintained by the ribonucleoprotein telomerase. Hsp90 facilitates the assembly of telomerase and remains associated with the functional complex, implying a direct involvement of Hsp90 in telomere length regulation. In an effort to elucidate the effects of Hsp90 inhibition on function and viability of human prostate cancer cells, both pharmacological (radicicol) and genetic (small interfering RNA) approaches were utilized to target Hsp90. Depletion of functional Hsp90 caused dramatic telomere shortening followed by apoptosis. Of particular significance, these cells exhibit a high level of nitric oxide synthase (NOS)-dependent free radical production, and simultaneous treatment of cells with the NOS inhibitor L-NAME resulted in telomere elongation and prevention of apoptosis. In addition, we observe significant DNA damage assessed by telomere dysfunction, although in the absence of a classical DNA damage response. Overall, our data suggest a novel mechanism whereby inhibition of Hsp90 disrupts free radical homeostasis and contributes directly to telomere erosion, further implicating Hsp90 as a potential therapeutic target for cancer cells.

Telomeres are complex protein-DNA structures found at the ends of vertebrate chromosomes. Telomeres act as a buffer for the gradual loss of chromosome ends that occurs as a result of incomplete DNA replication, which serves as a mitotic clock that controls cellular life span. Since telomeres are composed of many kilobases of noncoding repetitive sequences, these buffer sequences function as a protective mechanism to prevent the loss of genetic information stored on chromosomes. Most normal somatic cells have limited proliferative capacity and divide until critically shortened telomeres signal an irreversible growth arrest state known as cellular senescence (16, 30, 45). Senescence is also dependent upon functional checkpoint machinery, including p53 and pRB, inactivation of which leads to continued growth with further telomere shortening until the second proliferative barrier, known as crisis, occurs (14, 47). The rare cell capable of escaping crisis always activates a telomere maintenance mechanism, which typically involves the enzyme telomerase (6, 24).

Because of its nearly ubiquitous expression in human cancer, telomerase is an obvious chemotherapeutic target (40). Telomerase activity requires two core components, hTERT and hTR (10, 28, 46), to be assembled into a functionally active enzyme by the Hsp90 chaperone complex (20). We have previously demonstrated that chaperones are essential for optimal telomerase assembly in vitro (20) and that Hsp90 itself remains associated with the functional telomerase complex (11).

In a human prostate cancer model, increased assembly of telomerase by chaperones, including Hsp90, has been shown to correlate with prostate cancer progression, which is defined as increased aggressiveness in vivo (1). These findings indicate that increased expression of the Hsp90 chaperone complex with the associated activation of telomerase may be important steps in prostate cancer formation (1, 20). While telomerase in cancer progression has been widely studied (reviewed in reference 40), the role of chaperones in carcinogenesis and their interplay between telomerase and its substrate, the telomere, are less well defined.

Many studies indicate that Hsp90 chaperone inhibitors, such as geldanamycin (GA), 17-allylamino-17-demethoxy-geldanamycin (17-AAG), and radicicol (RAD), may be clinically useful as therapeutic agents for cancer patients (reviewed by 29, 13, 19). These inhibitors are capable of simultaneously targeting multiple Hsp90-associated proteins that are important in tumorigenicity, including N-ras, Ki-ras, HER-2, c-Raf-1, Akt, and mutant p53, ultimately resulting in the induction of cytostasis and/or apoptosis in cancer cells (21, 25, 41). Hsp90 is also involved in the production of free radicals from the nitric oxide synthase (NOS) pathway (27, 31, 34). Despite several studies describing the effect of chaperone inhibition on telomerase activity, few studies have examined the long-term consequences of Hsp90 inhibition on telomere length using either pharmacological or genetic approaches. Thus, the goal of our study was to establish a relationship between NOS-induced free radical production and telomere damage after genetic and/or pharmacologic disruption of Hsp90 function.

MATERIALS AND METHODS

Materials. Radicicol, geldanamycin, L-nitro-arginine methyl emide (L-NAME), and dimethyl sulfoxide (DMSO) were purchased from Sigma. 17-AAG was kindly provided by Neal Rosen (Memorial Sloan Kettering Cancer Center, NY).

Cell lines and isolation of subclones. All tumor cells were cultured in RPMI 1640 containing 5% fetal bovine serum and supplemented with ITS (insulin, 5 μg/ml; transferrin, 5 μg/ml; and selenium, 5 ng/ml; Collaborative Research),

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dexamethasone (0.1 μM), and gentamicin (0.05 mg/ml). The human prostate epithelial cell line, M12, used in these studies has been extensively characterized (1–3). All cells were mycoplasma free, as assessed by the mycoplasma T.C. Rapid Detection system (Gen-Probe, San Diego, CA). Isolation of subclones was achieved by seeding 500 cells onto a 15-cm^2 tissue culture plate and then expanding individual colonies after 2 weeks.

Design of siRNAs. Small interfering RNA (siRNA) sequences were designed according to the manufacturer's recommendations for use with the pSUPER.retro (*SUP*pression of *E*ndogenous *R*NA) system (Oligoengine). Briefly, a 19-nucleotide target sequence specific to the chaperone of interest was identified using Dharmacon siDESIGN center, and criteria were reviewed as described previously (5). Candidate sequences were used to synthesize a pair of 64-mer oligonucleotides. The complementary oligonucleotides were annealed according to the manufacturer's instructions prior to cloning into the pSUPER.retro vector. The 64-mer siRNA sequences were synthesized for both isoforms of Hsp90 (α and β) and the α isoform only, as follows: HSP90 (α and β), 5′-GATCCCCGT TT GAGAACCTCT GCAAATTCAA GAGATTTGCA GAGGTTCTCA AAC TTTTTGG AAA-3′; and HSP90α, 5′-AGCTTTTCCA AAAAGTTTGA GAA CCTCTGC AAATCTCTTG AATTTGCAGA GGTTCTCAAA CGGG-3′. The presence of the correct insert was confirmed by sequencing using the primers specific for p-SUPER.retro at positions 1242 to 1257 and its complement at positions 2645 to 2629.

Generation of retroviral cell lines and infection. The pSUPER.retro vectors were transfected using Fugene reagent (Roche) into the Phoenix A competent cell line, as recommended. The resulting retroviral supernatant was collected, filtered through a 0.45-μm filter, and incubated with M12 cells. Cells were allowed to recover before selection with 1 μg/ml puromycin, followed by isolation of individual clones as described above.

RAD treatments. For chronic RAD studies, M12 cells were seeded at 25% confluence and exposed to freshly prepared 0.3 μM RAD (1 mM stock in DMSO) or an equivalent volume of DMSO in standard RPMI culture medium. RAD-containing (or vehicle-containing) medium was replaced every 2 days. Cells were cultured and reseeded at 25% confluence, usually on the fourth day. At each passage, total cell numbers were determined, and population doublings were calculated using the formula [$\log_{10}$(number of cells counted/number of cells plated)]/0.3. All cells were kept in log-phase growth during the calculation of proliferation rates.

Terminal deoxynucleotidyltransferase-mediated dUTP-X nick-end labeling (TUNEL) assay. Cells were fixed directly on a six-well chamber slide in 4% formaldehyde for 10 min. Next, the cells were washed twice in phosphate-buffered saline (PBS) for 5 min each and fixed in acetic acid:ethanol (2:1) at −20°C for 5 min. Slides were washed, blocked in 1 mg/ml bovine serum albumin in PBS for 30 min at room temperature, and incubated with an enzyme mix containing 4 μl terminal transferase, 5× reaction buffer, 25 mM $CoCl_2$ and fluorescein 12-dUTP (Boehringer Mannheim) for 60 min at 37°C in a humidified chamber under light-sensitive conditions. Cells were washed twice in PBS for 5 min, mounted in Vectashield (Vector Labs), and stored at 4°C. Representative images were captured using an OLYMPUS IX70 fluorescence microscope (Optical Elements Corporation).

Immunoblotting. Cells grown in DMSO or radicicol (0.3 μM) or after stable integration of siRNAs were harvested at ~80% confluence, and proteins were isolated and electrophoresed as described previously (8). Nitrocellulose membranes were subjected to immunoblotting as described previously (8) with anti-Hsp90, anti-p23, (generous gifts of David Toft, Mayo Clinic, Rochester, MN; all 1:5,000 dilution), antiactin (Sigma) (1), antiubiquitin (Stressgen, Vancouver, British Columbia), or anti-universal NOS (anti-uNOS; Stressgen) (1:1,000), followed by incubation with secondary antibody. Detection involved the use of a Pierce ECL kit with exposure to Kodak X-OMAT film.

Measurements of superoxide generation. Dihydroethidium (DHE) (Sigma) is a cell-permeative dye that is oxidized to fluorescent ethidium bromide by superoxides and intercalates into DNA. Prior to addition of DHE, cells were thoroughly rinsed to remove all traces of drug and then incubated in fresh medium containing 0.5 μM DHE for 30 min at 37°C in the dark. Intact cell images were captured by fluorescence microscopy. Alternatively, cells were treated as described above except that after incubation in dye, cells were trypsinized, washed, and resuspended in PBS at 1×10^6/ml for analysis using flow cytometry.

Telomeric repeat amplification protocol (TRAP assay). Telomerase activity was measured according to the manufacturer's instructions using the TRAP-eze detection kit (Serologicals, Purchase, NY), as described previously (1, 11). Relative telomerase activity was quantitated by ImageQuant software (Molecular Dynamics) analysis of the ratio of the telomerase ladder to the included 36-bp internal standard.

TALA. A telomere amount and length assay (TALA) was modified from the work of Gan and colleagues (12). Genomic DNA was digested using six restriction enzymes, AluI, MspI, RsaI, CfoI, HaeIII, and HinfI (Gibco-BRL). A radiolabeled telomere-specific probe, $(TTAGGG)_4$, was added to digested genomic DNA, followed by hybridization for 2 h at 55°C. Samples were cooled to 4°C, loaded onto a 0.8% agarose gel, and electrophoresed for 18 h. Dried gels were wrapped in plastic and exposed to phosphorimaging cassettes. Average telomere lengths were determined as described previously (32).

Cytogenetic analysis. Standard chromosomal harvest procedures were used with a colcemid time of 2 h (37). Metaphase chromosomes were visualized using conventional Giemsa staining (4), and the chromosomal changes that were observed were tallied. Metaphase spreads ($n = 50$) were scored for chromosomal findings from the DMSO- and RAD-treated M12 cell lines at day 59 after treatment.

RESULTS

Telomere erosion after pharmacologic inhibition of Hsp90. The molecular and cellular effects of chronic inhibition of Hsp90 function were evaluated with the M12 metastatic prostate cancer cell line (2, 3). Notably, M12, like many human cancer cells, expresses high levels of Hsp90 (1), making this cell line an ideal model with which to study chaperone inhibition. GA and its analog 17-AAG are benzoquinone ansamycin antibiotics that are commonly used to study Hsp90 function and are currently in clinical trials as therapeutic cancer drugs (29). A second class of Hsp90 inhibitor, RAD, is a macrocyclic antifungal antibiotic that appears less toxic to the M12 prostate cancer cell line and was better suited for chronic Hsp90 inhibition studies. All of these pharmacological agents bind to the same amino-terminal ATP binding pocket on Hsp90 and disrupt function by preventing ATP hydrolysis, which is an essential process required for chaperone activity.

Traditional studies using Hsp90 inhibitors utilize an acute treatment, assessing the cellular effects in the short term (29). To determine the long-term consequences of Hsp90 inhibition after chronic exposure to the less-toxic RAD compound, M12 prostate cancer cells were cultured in medium containing a low concentration (0.3 μM) of RAD for 60 days. DNA was isolated periodically from vehicle (DMSO)-treated or RAD-treated M12 cells, and telomere lengths were analyzed. We found that telomeres remained at a constant level ranging between 3.5 and 4.0 kb in the presence of vehicle, while cells treated with RAD undergo a gradual telomere shortening starting at ~20 days, continuing throughout the course of treatment (Fig. 1A). Average telomere lengths quantified from multiple assays indicate that telomeres shorten from ~3.75 kb to ~1.5 kb during chronic RAD treatment, equating to a shortening rate of ~200 bp per population doubling. Although chronic treatment of M12 cells at 0.3 μM RAD could be achieved without effects on cell viability/toxicity in the short term, continued treatment resulted in a delayed induction of cell death after ~55 days ($n = 6$; death reproducibly occurred between days 54 and 60) (Fig. 1B). We found that nearly 100% of the treated cells undergo apoptosis as assessed by TUNEL (Fig. 1C). Cells treated with vehicle are TUNEL negative (Fig. 1C), suggesting that critically short telomeres in cells chronically treated with RAD signal the activation of the apoptotic cascade.

To characterize the mechanism underlying the induction of telomere erosion and delayed apoptosis, we analyzed the effects of chronic RAD treatment on telomerase activity. RAD treatment reduced telomerase activity as expected, but telo-

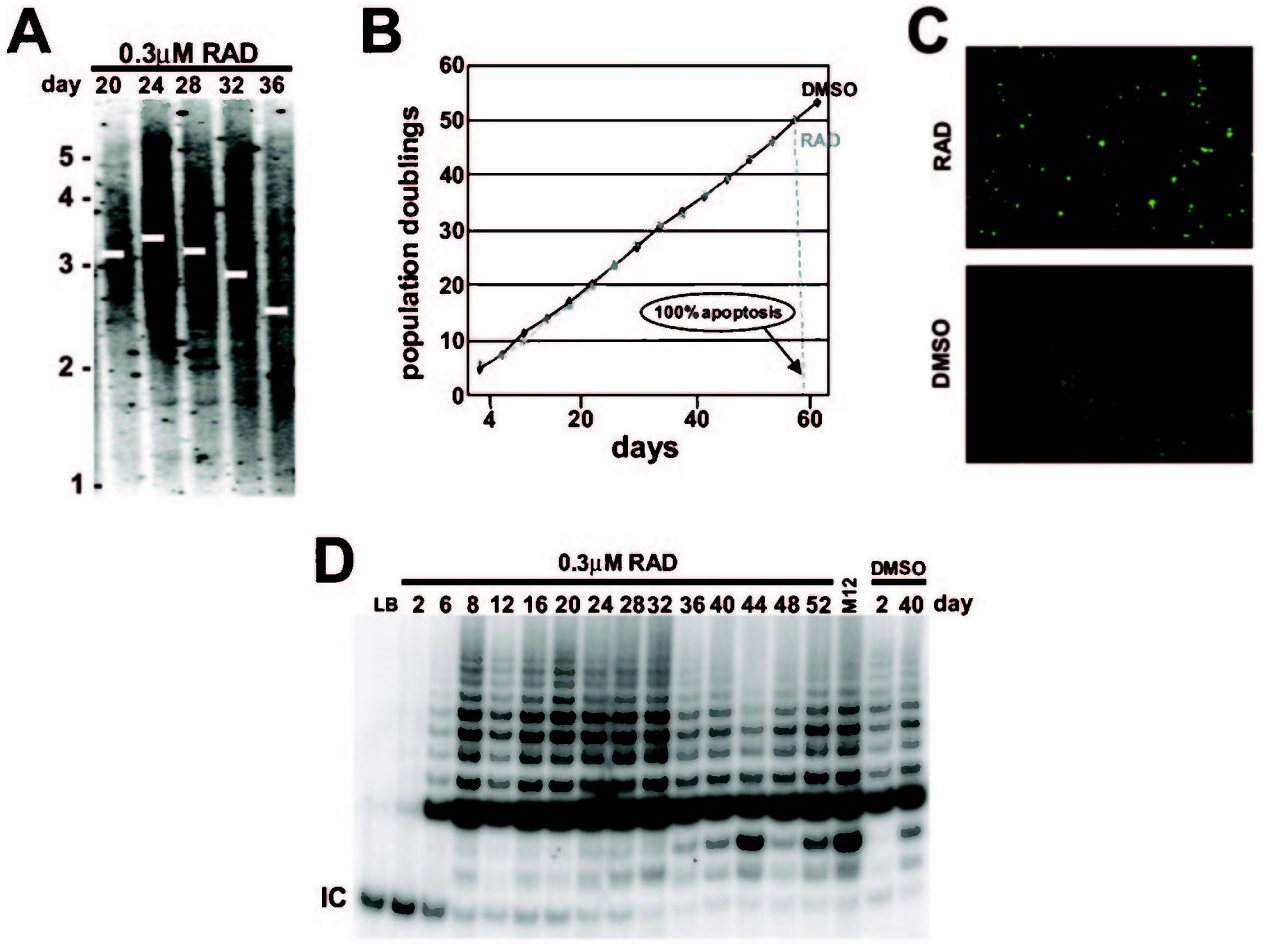

FIG. 1. Treatment of prostate cancer cells with radicicol transiently inhibits telomerase activity and reduces telomere length, culminating in apoptosis. (A) Genomic DNA was isolated from cells exposed to chronic RAD and digested with six restriction enzymes. The digested DNA was hybridized with a ^{32}P-labeled telomere probe, and telomere signals separated by gel electrophoresis. A size ladder in kilobase is noted on the left of the blot. (B) Cells continuously cultured in 0.3 μM RAD or vehicle (DMSO) were assessed for cell growth in log phase over the course of treatment. (C) Following chronic RAD treatment for 55 days, cells were stained with terminal transferase and fluoroscein 12-dUTP (TUNEL) and 4′,6′-diamidino-2-phenylindole (not shown). All 4′,6′-diamidino-2-phenylindole-positive RAD-treated cells were stained with TUNEL, visualized by an intense fluorescent (green) signal, while the DMSO controls were not TUNEL positive. (D) The M12 prostate cancer cells treated with 0.3 μM radicicol or DMSO were periodically analyzed for telomerase activity (100 cell equivalents) by the TRAP assay. IC is the 36-bp internal PCR control.

merase inhibition was only transient, and after 1 week, activity was restored (Fig. 1D). Furthermore, the level of telomerase activity detected after 8 days of RAD treatment was comparable to, if not higher than, that in untreated M12 cells or cells treated with vehicle. In fact, all three Hsp90 inhibitors were capable of transiently inhibiting telomerase activity at concentrations that did not affect cell viability (data not shown). Additionally, we repeated these experiments in an immortalized prostate cell line, P69 (2, 3), with nearly identical results, showing a transient inhibition of telomerase activity, a decreased telomere length over the course of treatment, and induction of apoptosis within 30 days (data not shown). The likely explanation for the accelerated cell death in the P69 cell line may have to do with its telomere lengths, which are significantly more heterogeneous than those of the M12 cells (data not shown). Together, these data suggest a mechanism of telomere erosion independent of telomerase inhibition.

Inhibition of Hsp90 results in the ubiquitination of a number of its bound target proteins, which are then targeted to the ubiquitin-mediated proteasome degradation pathway (29). To determine if the cellular response to RAD remains constant throughout the course of treatment (i.e., constant Hsp90 inhibition), cells were analyzed for global changes in the levels of ubiquitinated proteins. Chronic RAD treatment increases the levels of ubiquitinated proteins compared to those in cells treated with vehicle (data not shown), and those levels remain constant throughout the course of treatment, indicating that Hsp90 function remains inhibited in RAD-treated cells.

Free radical production in cells chronically treated with radicicol. The observation that telomeres shorten in the presence of detectable telomerase activity may at first appear contradictory to our understanding of how telomerase maintains telomeres. We therefore set out to establish the mechanism of telomere shortening in RAD-treated cells. Interestingly, inhibition of Hsp90 by GA, 17-AAG, and RAD has been impli-

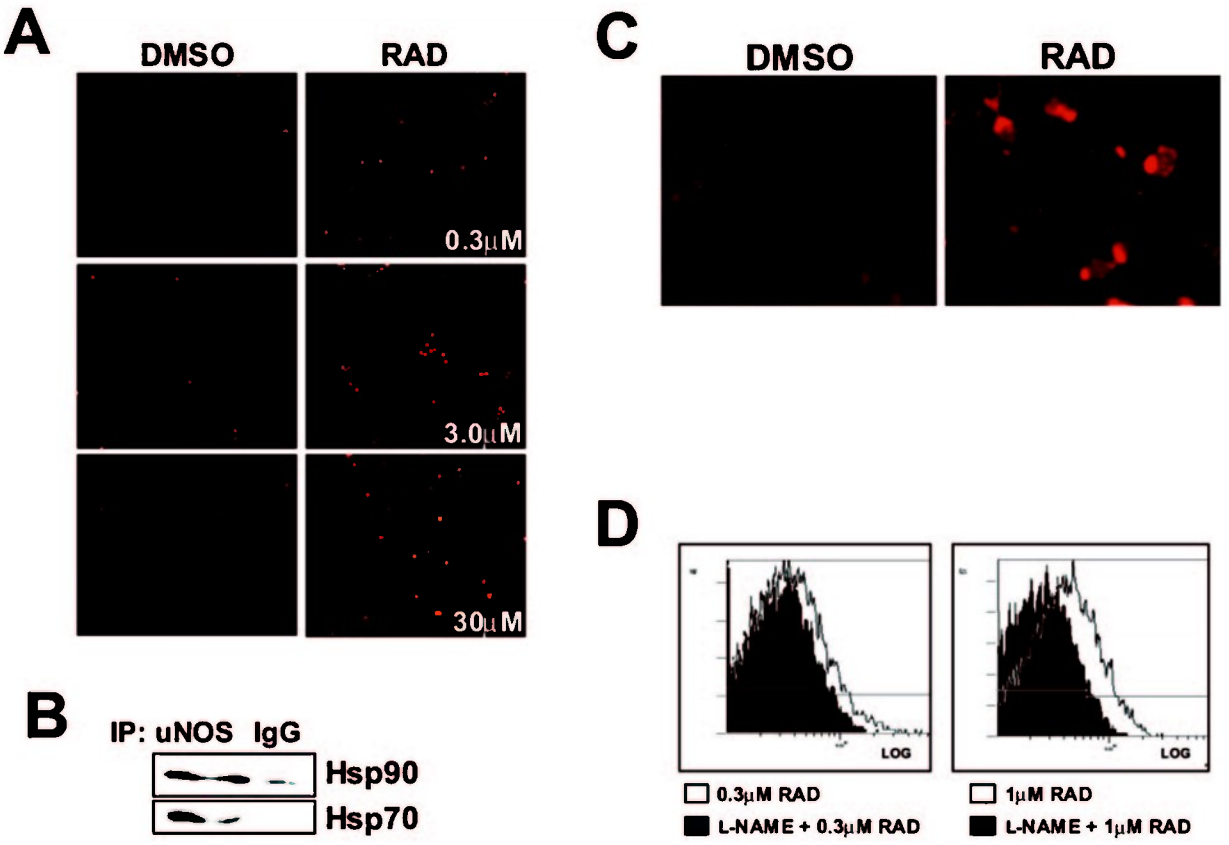

FIG. 2. Radicicol induces NOS-dependent free radicals. (A) RAD induces a dose-dependent increase in superoxide (O_2^-) in the M12 cells. Cells were incubated in fresh medium containing DHE for 30 min, followed by imaging using a fluorescence microscope. Cells generating high concentrations of free radicals emit an intense red fluorescence. (B) Hsp90 and Hsp70 were immunoprecipitated using a NOS-specific antibody or with control immunoglobulin G antibodies. Following immunoprecipitation, proteins were transferred to nitrocellulose and immunoblotted for either Hsp90 or Hsp70 to determine association with NOS. (C) Cells chronically treated with RAD or DMSO for 55 days were reseeded into eight-well chamber slides, incubated with DHE, and visualized. (D) Cells were incubated in 0.3 μM or 1 μM RAD for 24 h with or without 500 μM of the NOS inhibitor L-NAME, and then incubated in fresh medium containing DHE. Free radical production was quantified by flow cytometry analysis and plotted on a logarithmic scale.

cated in the production of oxidative free radicals by disruption of the NOS pathway (31, 42). Therefore, we asked whether RAD treatment might induce oxidative free radicals that contribute to the telomere shortening.

M12 cells were exposed to various concentrations of RAD for 24 h, washed free of excess drug, and incubated with 5 μM DHE, a reduced form of ethidium dye that is oxidized by reactive oxygen species (ROS) to fluorescent ethidium, which incorporates into DNA. Cells treated with increasing concentrations of RAD have a concentration-dependent increase in ROS levels (Fig. 2A), which indicates that chronic treatment of RAD could lead to an accumulation of DNA damage induced by a constant exposure to oxidative free radicals. We then confirmed that chronic Hsp90 inhibition by RAD caused an increase in ROS generation (Fig. 2C), as reflected by more-intense DNA staining than in the short-term experiments (Fig. 2A).

To determine if Hsp90 interacts with NOS in the M12 cells, we used a universal NOS antibody to coimmunoprecipitate NOS-associated proteins and immunoblotted with antibodies specific to Hsp90 and Hsp70. Both Hsp90 and Hsp70 were associated with NOS before and after treatment with RAD (Fig. 2B), consistent with the notion that chaperone inhibition does not alter Hsp90's ability to bind its target proteins.

Production of free radicals and telomere shortening are dependent on nitric oxide synthase activity. In previous studies, investigators demonstrate that the production of free radicals by Hsp90 inhibitors originates from the uncoupling of NOS enzymatic function (31). To demonstrate that free radical production in our system was derived from uncoupling of NOS, cells were incubated with 1.0 μM or 0.3 μM RAD for 24 h, followed by 1 h with the NOS inhibitor L-NAME (500 μM). L-NAME completely inhibits production of free radicals and nitric oxide, rendering the NOS enzyme functionally inactive. Simultaneous incubation of RAD with the NOS inhibitor L-NAME caused a shift in the DHE staining intensity as quantified by flow cytometry, corresponding to a reduction in ROS production compared to that with RAD treatment alone (Fig. 2D; also data not shown).

To determine whether the cells (and their telomere lengths) could recover from chronic RAD treatment, cells were cultured for 49 days with 0.3 μM RAD, and on day 50, drug was removed or treatment continued. While the cells maintained in

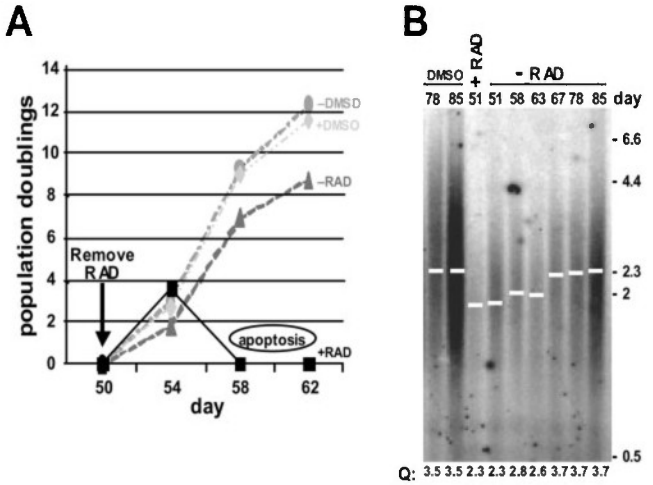

FIG. 3. Telomere regrowth and prevention of apoptosis in cells after radicicol is removed. (A) Growth of M12 cells continuously cultured in the presence of RAD for 49 days. At day 50, cells were either maintained in RAD medium (or DMSO) or removed from drug (or vehicle control). Growth was calculated as population doublings from logarithmically dividing cells. (B) RAD was removed at day 49, and cells were cultured for the indicated numbers of days beyond drug (or vehicle) removal. The average telomere length (Q) was measured after the TALA procedure using ImageQuant software as described previously (32), with the size ladder in kilobases to the right of the blot. Note the increase in mean telomere length after RAD removal, as indicated by the quantitation below each lane.

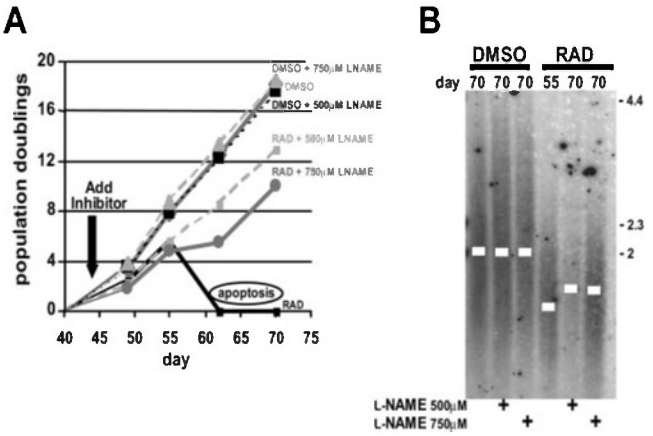

FIG. 4. Inhibition of the NOS pathway prevents telomere shortening and apoptosis in RAD-treated cells. Cells were cultured for 44 days in RAD medium. On day 45, cells were either maintained in RAD alone (or DMSO) or cocultured in L-NAME (or DMSO) as indicated. Cells were assessed for (A) growth over time and (B) telomere length, determined using TALA. Three plates were simultaneously treated and counted to give an average growth rate. Size ladders are noted to the right of the blot in kilobase pairs (B).

RAD consistently underwent apoptosis between days 55 and 60, as shown in Fig. 1C, cells removed from RAD continued to proliferate for more than 35 doublings beyond this point (Fig. 3A), with growth rates that were not appreciably different from those of DMSO-treated cells.

We also hypothesized that telomere lengths were maintained or elongated in the absence of drug and that telomerase would be capable of restoring telomere length after RAD removal. Telomere lengths of DMSO-treated cells were significantly longer than those of RAD-treated cells (Fig. 3B). However, continued culture of cells with shortened telomeres without RAD resulted in gradual telomere lengthening, suggesting that telomerase is more efficient at maintaining telomeres in the absence of RAD-induced free radicals. Furthermore, this telomere elongation protected cells from apoptosis. Thus, removing cells from RAD treatment appears to prevent further free radical production, protecting cells from further telomere damage and induction of cell death.

To determine if inhibition of NOS would also prevent telomere shortening and delayed apoptosis in RAD-treated cells, cells were treated chronically for 44 days, and on day 45, cells were maintained in RAD (or DMSO) with or without the NOS inhibitor L-NAME. Cells treated with RAD alone died as expected, while cells cocultured with L-NAME continued to proliferate well beyond the point at which RAD-treated cells underwent apoptosis (Fig. 4A). Telomere lengths in control cells (DMSO or DMSO/L-NAME) were similar to lengths in untreated cells. While chronic RAD treatment resulted in very short telomeres, telomere lengths of cells treated with the combination of RAD and L-NAME appeared to be maintained (Fig. 4B). Importantly, telomere lengths did not continue to shorten, suggesting that the shortest telomeres may be maintained in order to prevent cell death. This observation that simultaneous treatment with RAD and NOS inhibitors prevents free radical production and cell death directly implicates NOS as the source of the telomere damage. Thus, it appears that without constant damage of telomeres by free radicals, a functional telomerase is capable of at least maintaining telomeres, thereby preventing apoptosis.

Genetic inhibition of Hsp90 function. The novel finding that Hsp90 inhibition results in the gradual loss of telomere length and eventually delayed apoptosis suggests that an accumulation of free radical-induced telomere damage may occur over time. Because of concerns of specificity and potential RAD–L-NAME interactions, we chose to address the mechanism of telomere erosion and free radical homeostasis genetically by creating stable cell lines expressing siRNA hairpin constructs that specifically target Hsp90 mRNA.

Hsp90 exists in two isoforms encoded on separate genes, referred to as Hsp90α and -β for human cells (35). In humans, Hsp90β is constitutively expressed and moderately inducible, whereas Hsp90α is generally expressed at low basal levels that are induced dramatically in response to stress (13). For Hsp90 siRNA constructs, target sequences were selected in a region that is conserved in all members of the Hsp90 family. In addition, a similar siRNA was created to specifically target only the Hsp90α isoform. While there is evidence to suggest some overlap in function (33, 48), there are also some Hsp90 isoform-specific functions as well (9).

M12 prostate cancer cells were infected with vector (pSUPER), the Hsp90 siRNA (targets both Hsp90α and -β), or the Hsp90α siRNA, and expression of Hsp90 and Hsp90α was monitored by immunoblotting. Compared to empty vector controls, those cells stably expressing the α/β Hsp90 siRNA had a considerable reduction of total Hsp90 protein expression (Fig. 5A), while cells expressing the Hsp90α siRNA showed only a reduction in Hsp90α (Fig. 5B). Single-cell-derived

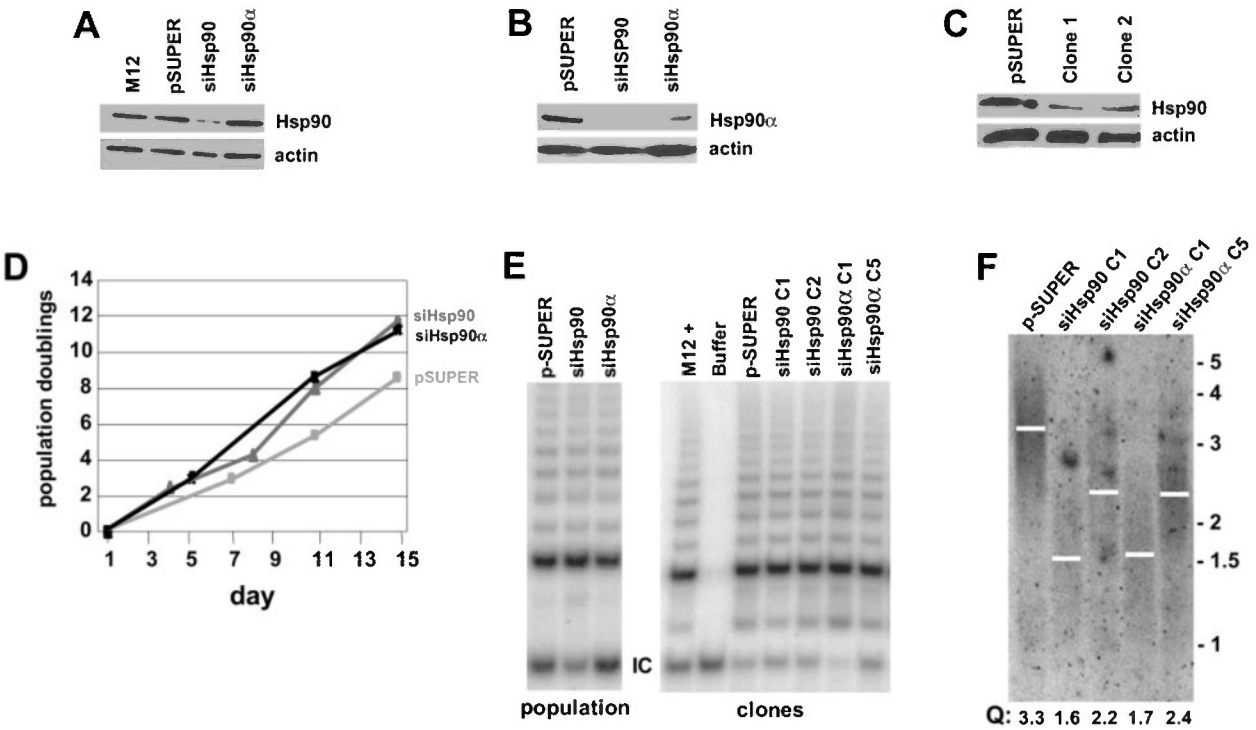

FIG. 5. Genetic inhibition of Hsp90 causes telomere shortening without altering telomerase activity or cell growth. (A) Mass cultures of M12 cells with siRNA expression specific for either Hsp90α or both Hsp90 isoforms (α and β), along with vector (pSUPER) controls, were tested for expression of Hsp90 by immunoblotting. (B) Hsp90α protein levels following stable integration of the Hsp90 and Hsp90α siRNA constructs. (C) Hsp90 expression in single-cell-derived clones (1 and 2) after infection with siRNA directed at both Hsp90 isoforms. (D) Growth of logarithmically cultured M12 cells following Hsp90 siRNA expression was calculated as an average for three separate plates, and overall growth was based on three independent experiments. (E) Telomerase activity was tested in M12 mass cultures (left panel) and clones (right panel) following infection with siRNA constructs directed at Hsp90. Five hundred cell equivalents were analyzed for telomerase activity by the TRAP assay. C1/C2 means clone 1 or clone 2. (F) Telomere lengths of individual clones were analyzed using 20 μg of genomic DNA and the TALA protocol as described for Fig. 1. Average telomere length (Q) was measured as described previously (32).

clones were isolated (6 to 12 for each siRNA type), and representative clones with at least an approximately 50% reduction in Hsp90 protein abundance (both for the α/β Hsp90 siRNA and for the Hsp90α siRNA) were used in the remainder of these studies (Fig. 5C). Since we were unable to isolate individual clones with much less than 50% reduction in Hsp90 expression, this is presumably the minimum level of Hsp90 chaperone activity required to maintain cell survival.

Populations of cells infected with Hsp90α constructs did not appear to have an observable change in the total Hsp90 (Hsp90α and -β) chaperone expression level (Fig. 5A). However, the expression of the Hsp90α isoform was dramatically reduced (Fig. 5B). This observation is not unexpected, since it is an immunoblot for total Hsp90, which makes up 2 to 4% of total cellular protein, and small changes in the weakly expressed Hsp90α isoform were expected to be masked by the predominant Hsp90β isoform (13).

Cells stably infected with siRNA constructs targeting Hsp90 displayed no notable changes in proliferation, indicating that cells can survive with reduced expression of these chaperones without any observable cellular toxicity (Fig. 5D). Furthermore, there were no measurable changes in telomerase activity between populations of cells or individual clone samples that expressed siRNA inserts and empty vector controls (Fig. 5E). Creation of cell lines that stably express siRNA-like constructs requires a 5- to 7-day antibiotic selection process, followed by analysis by immunoblotting and/or TRAP. Therefore, it is likely that a transient inhibition of telomerase activity, like that observed in drug-treated cells between days 2 and 6, would be missed in siRNA cells due to the time frame of the selection process. Taken together, the data from both the drug and genetic inhibition of Hsp90 studies described above leads to the conclusion that there are no long-term effects on telomerase activity in the presence of reduced functional Hsp90.

Telomere length analysis of the siRNA cells confirmed the findings using RAD, indicating that reduction in Hsp90 function, whether global Hsp90 or Hsp90α, leads to telomere shortening (Fig. 5F). The extent of shortening was similar in both Hsp90 siRNA-infected cells and chronic RAD-treated cells, with a reduction from 3.3 kb down to 1.5 to 2 kb (Fig. 5F). It has previously been shown that there is significant clonal heterogeneity in terms of telomerase activity and telomere length in established cell lines (38), with a range of longer and shorter telomeres in single-cell-derived clones. While we show data for only four clones, all of the clones analyzed had shorter telomeres than the parental line (data not shown), indicating that telomere shortening has occurred in the Hsp90 siRNA cell lines.

NOS-dependent free radical production induces telomere shortening after genetic inhibition of Hsp90. The similarity

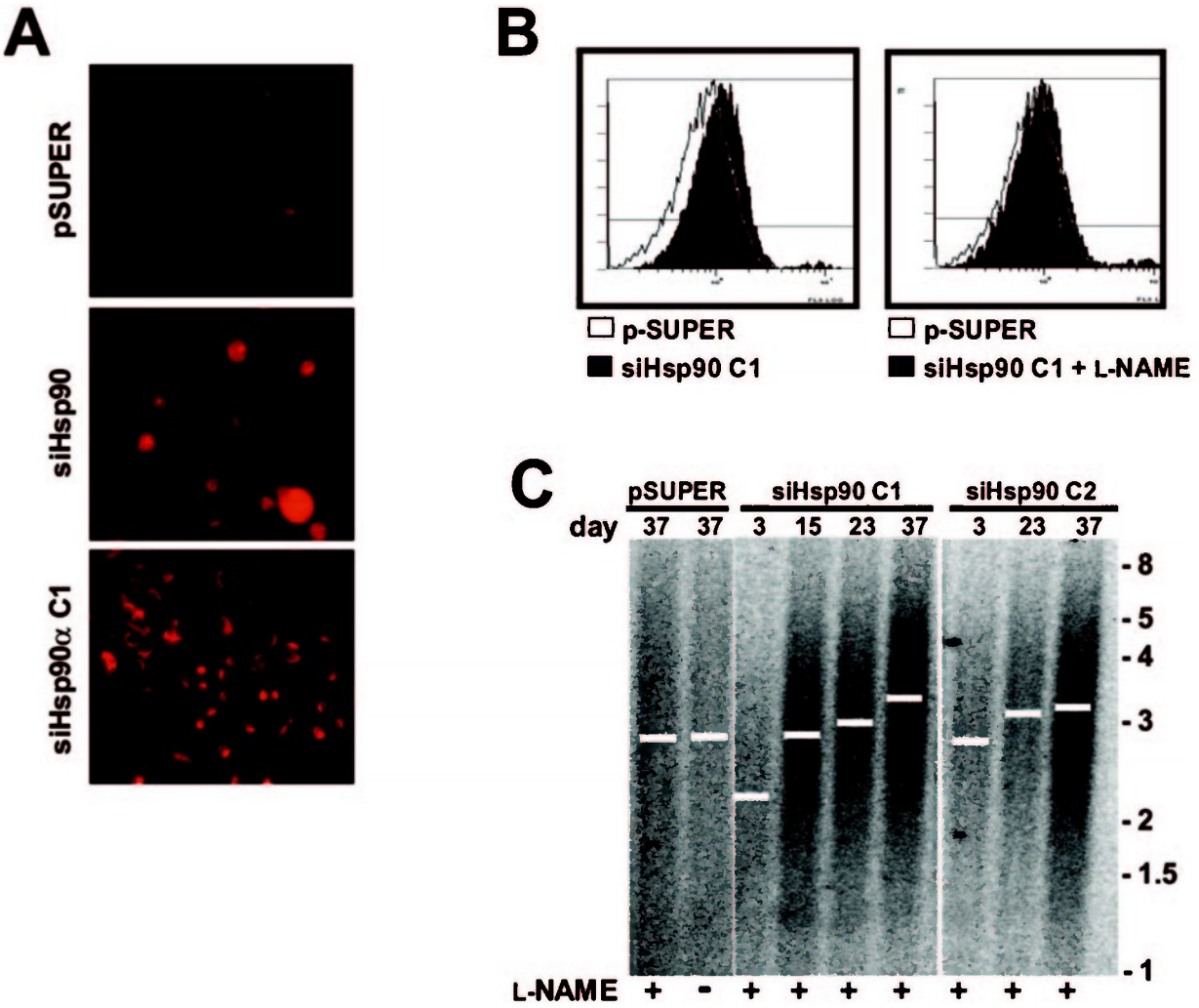

FIG. 6. Inhibition of NOS-induced free radicals prevents telomere erosion. (A) Cells expressing the vector (pSUPER) or siRNAs against total Hsp90 or Hsp90α were incubated in medium containing DHE. Increased superoxide levels are visualized by an intense red fluorescence. (B) Hsp90 siRNA-expressing cells were incubated in fresh medium (or medium containing L-NAME for 24 h) and then with medium containing DHE. Free radical production was analyzed by flow cytometry. (C) Telomere lengths (TALA) were analyzed over time in cells expressing Hsp90 siRNAs and compared to those of the pSUPER controls after incubation with 500 mM L-NAME.

between pharmacological and genetic (siRNA) inhibition of Hsp90 function on growth, telomerase activity, and telomere length points to a common mechanism of action that is responsible for induction of telomere shortening in these cells. Given that we observed increased NOS-dependent free radical production combined with telomere shortening following chronic RAD exposure, cells were similarly evaluated following genetic inhibition of Hsp90 for the production of free radicals. As shown with pharmacological inhibition of Hsp90, genetic knockdown of Hsp90 resulted in elevated ROS production above levels observed in cells with an empty vector (Fig. 6A and 6B, right panel). Production of free radicals is reduced after a 1-h incubation with the NOS inhibitor L-NAME (data not shown) and completely blocked after a 24-h incubation (Fig. 6B, left panel).

Since both pharmacological and genetic inhibition of Hsp90 resulted in NOS-dependent free radical production, we addressed the question of whether telomere length in cells expressing siRNAs directed at Hsp90 can be maintained or even elongated if ROS production is inhibited by continuous culture with the NOS inhibitor L-NAME. Telomere length analysis in cells expressing Hsp90 siRNA constructs indicates that L-NAME allows for an increase in telomere lengths (Fig. 6C), consistent with the telomere elongation observed with both RAD and L-NAME (see Fig. 4B) and as dramatic as that observed in RAD-treated cells upon removal from drug treatment (see Fig. 3B). These data imply that a certain minimum level of Hsp90 is required by cells to maintain essential intracellular functions. Inhibition of Hsp90 results in an inability to maintain proper cellular homeostasis, causing a range of altered physiological responses including an imbalance of free radical production and telomere damage.

DNA damage and telomere dysfunction. To conclusively show that DNA damage does occur in these cells, we determined the level of acquired chromosomal changes present in RAD-treated cells versus vehicle (DMSO)-treated cells, as

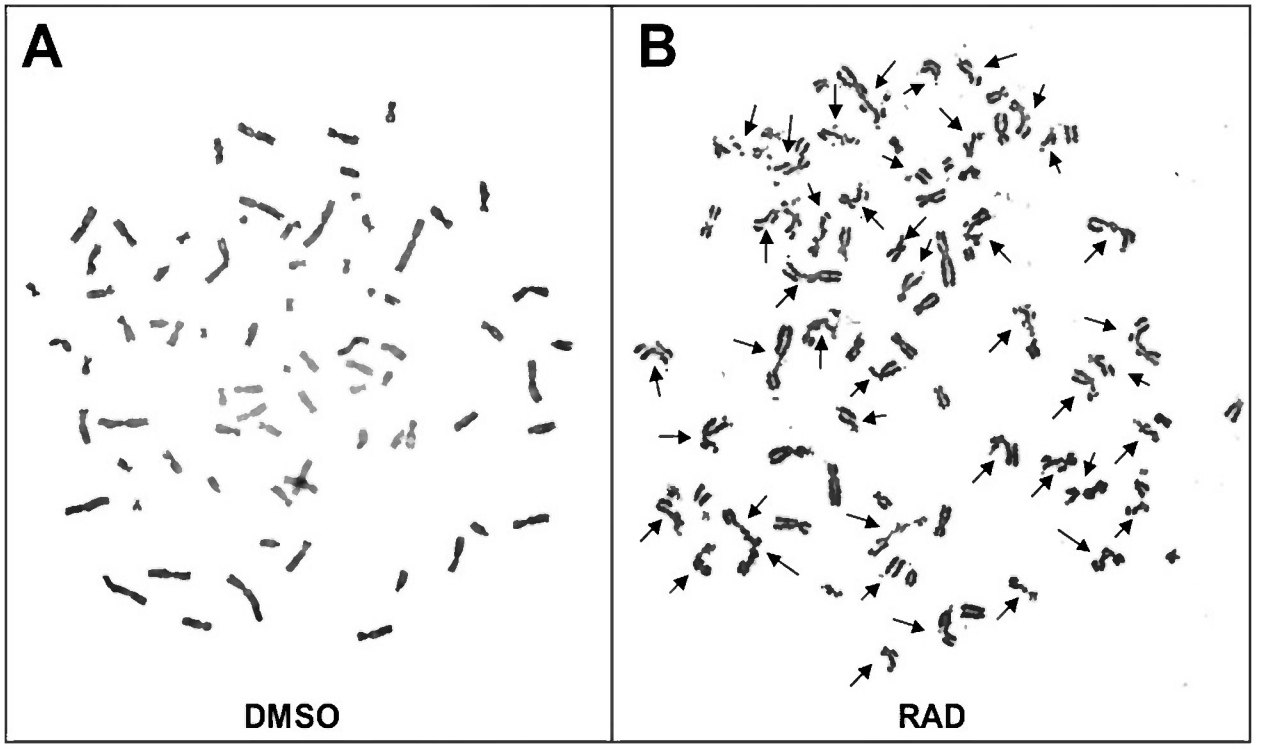

FIG. 7. Chromosomal damage and telomere dysfunction induced by inhibition of Hsp90 and production of free radicals. Cells were harvested after 59 days of treatment and subjected to a 2-h colcemid block to assess metaphase chromosomes. Metaphase spreads were evaluated from DMSO-treated (A) and RAD-treated (B) M12 cells after 59 days of treatment. The DMSO cell chromosome numbers ranged from near diploid with few structural abnormalities to near triploid (A), while the RAD-treated cells were predominantly near triploid. The M12-treated cells show many cytogenetic abnormalities related to telomere dysfunction, including end associations/fusions and end breaks as indicated by the arrows.

previously done (8). Figure 7 shows representative metaphase spreads clearly demonstrating numerous telomere-specific chromosomal abnormalities in the RAD-treated cells compared to the DMSO-treated cells, the data for which are summarized in Table 1. The M12 RAD-treated cells presented in a continuum, with the least-affected cells having a small number of end breaks and/or end fusions, progressing to a large number of end fusions/breaks along with interstitial breaks, progressing to massive chromatin disintegration. Based on the significantly increased frequency of end breaks and end fusions, one can safely conclude that there is damage at the telomere in these treated cells.

DISCUSSION

Our data support a model in which Hsp90 regulates the production of nitric oxide ($^{\cdot}NO$) and superoxide (O_2^{-}) through NOS, depending partially on the functional status of Hsp90 and its ability to uncouple NOS enzymatic activity (31). A consequence of Hsp90 deregulation is that NOS promotes the production of free radicals, causing extensive DNA damage, which, as our data show, preferentially targets telomeres (Fig. 8).

Hsp90 inhibitors indirectly induce telomere shortening and transiently inhibit telomerase. Several reports have described the use of direct telomerase inhibition as a potential adjuvant to traditional cancer therapies and to prevent metastasis and recurrence of residual disease after traditional forms of cancer treatment (15, 18, 39). Our goal was to determine if Hsp90 inhibition could be used to indirectly target telomerase, which is particularly appealing because there are several established antibiotic inhibitors of Hsp90 that are well tolerated in humans and are currently in clinical trials as potential cancer therapies (23, 29). Although there were only limited and transient effects on telomerase activity, our results are consistent with those of others describing indirect targeting of telomerase in colon adenocarcinoma and melanoma cell lines, using benzoquinone antibiotics (21, 43). Here, we show that a functionally related but different class of antibiotic, radicicol, was also capable of transiently inhibiting telomerase at low doses without detrimental effects on viability but that chronic inhibition of Hsp90 results in telomere shortening and ultimately in cell death through the generation of reactive oxygen via deregulation of the NOS pathway.

It is interesting that telomerase activity recovers in cells during chronic Hsp90 inhibition, suggesting telomerase assembly in the absence of Hsp90 function. It may be that Hsp90 is not strictly required for assembly of extractable telomerase but is necessary for telomere elongation, consistent with our previous findings that Hsp90 is associated with the functional telomerase enzyme (11). Alternatively, and perhaps more simply, because telomerase is a low-abundance Hsp90 target, it may also be that there is enough residual functional Hsp90 in cells to fully assemble active telomerase. Recent evidence suggests that the TRAP assay for assessing telomerase activity

TABLE 1. Chromosomal abnormalities after radicicol treatment in M12 prostate cells

Chromosomal characteristic	Value for cell line on day 59	
	M12 (DMSO)	M12 (RAD)
No. of metaphases scored	50	50
Chromosome no.		
No. of metaphases that are near diploid	25	11
Range (modal number)	39–54 (42)	37–54 (45)
No. of metaphases that are near triploid	22	30
Range (modal number)	67–82 (76)	58–80 (76)
No. of metaphases with ≥100 chromosomes	1	6
No. of metaphase spreads too fragmented to score	0	3
No. of metaphases with abnormalities	14	48
No. of end fusions (range of no. of chromosomes in fusions/cell)	4 (2–3)	38 (2–53)
No. of rings	0	0
No. of radials	0	0
No. of end breaks (range of no. of breaks/cell)	4 (1)	32 (1–15)[b]
No. of dicentrics	0	0
No. of interstitial chromatid breaks (range of no. of breaks/cell)	7 (1)	26 (1–10)[b]
No. of interstitial chromosome breaks	0	—[b]
No. of small "fragments" (range of no. of markers/cells)	3 (1–2)	24 (1 to too numerous to accurately count)
No. pulverized[a]	0	3

[a] Pulverized chromosomes are those that have lost morphology and appear to have their chromatin broken (disintegrating). Since distinct chromosomal anomalies could not be seen, these are not included in the total number of abnormalities.

[b] In several metaphase spreads, the breakage was too extensive to allow an accurate count. Thus, the numbers shown are the most conservative scores of breakage that could clearly be categorized.

levels may not be sufficiently sensitive to detect the subtle changes in telomerase activity/function observed by Hsp90 inhibition (26, 44), so that even though extractable telomerase activity is abundant, telomerically functional telomerase may be significantly reduced.

Inhibition of Hsp90 disrupts free radical homeostasis. Increasing evidence indicates that Hsp90 is intimately involved in the production of oxidative damage as a result of its interaction with NOS. Hsp90 functionally associates with NOS, an enzyme involved in converting L-arginine into L-citrulline and nitric oxide (·NO) (27). These studies demonstrate that NOS, in addition to producing ·NO, is also capable of producing O_2^- free radicals and H_2O_2 (34). In particular, inhibitors of Hsp90, including GA, 17-AAG, and RAD, can disrupt NOS activity, resulting in an increase in NOS-dependent O_2^- radicals (31). We show that pharmacologic and genetic inhibition of Hsp90 results in significant free radical production and that a specific NOS inhibitor (L-NAME) blocks ROS production. Taken together, these results clearly demonstrate a direct link between DNA damage induced by inhibition of Hsp90 and deregulation of the NOS pathway.

NOS-induced free radical production damages telomeres. It is well established that oxidative free radicals can damage DNA, including telomeric DNA. When combined with the finding that RAD induces high levels of ROS, these two observations suggest that the increased production of free radicals by Hsp90 inhibition may contribute to telomerase-independent telomere damage. Furthermore, several lines of evidence suggest that telomeres may act as preferential targets for free radical damage. When telomeric oligonucleotides were exposed to oxidative stress using H_2O_2 and O_2^-, DNA damage (i.e., cleavage sites and adducts) preferentially occurred at the 5′ site of 5′-GGG-3′ sequence in an oligonucleotide (22). Fenton reactions (between H_2O_2 and Fe^{2+}) cause preferential cleavage and strand breaks located at the 5′ end of the sequence RGGG in a plasmid containing 81-telomere repeats (17). They propose that telomeric DNA may protect the genome from DNA damage by attracting oxidative damage to noncoding telomeric sequences (17), which seems plausible given the telomere's repetitive, G-rich sequence and its overall structure. These studies together with our current data allow us to predict that the extent of telomeric damage induced by a lack of functional chaperones is greater than the compensatory mechanisms of telomerase, since detectable telomerase activity failed to protect against telomere shortening in cells with inhibited Hsp90. Clearly, our cytogenetic analysis shows significant chromosome damage of the type consistent with telomere dysfunction, suggesting high levels of telomere damage after treatment with RAD. In an attempt to characterize the DNA damage response, we assessed for the binding of γ-H2AX and 53BP1 at the telomeres after treatment with RAD, since these proteins have been shown to be first responders to DNA damage at both interstitial and telomeric sites (7). However, we were unable to show a classic DNA damage response in the RAD-treated cells, even though treatment with γ-irradiation resulted in classic DNA damage-induced foci (data not shown) (7). Because radicicol is not a DNA damaging agent, since it

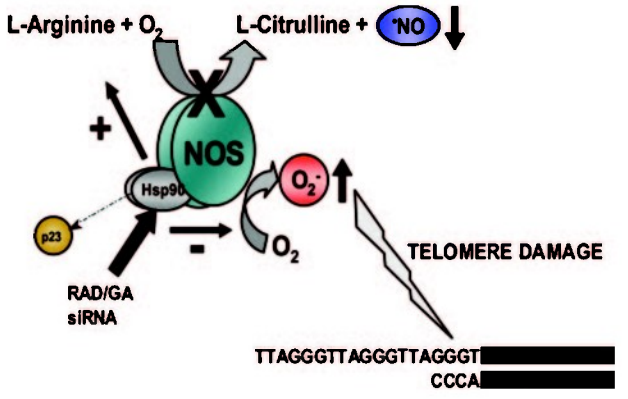

FIG. 8. Model of Hsp90-mediated regulation of NOS and free radical-induced telomere damage. NOS produces nitric oxide (·NO) as a by-product of the reaction in which L-arginine and oxygen are converted to L-citrulline. Hsp90 association with NOS promotes this conversion and suppresses the pathway by which NOS generates the superoxide (O_2^-) free radicals. Inhibition of Hsp90 function pharmacologically (GA or RAD) or genetically (siRNA) disrupts NOS conformation to inhibit ·NO production and promote O_2^- generation, which results in the accumulation of telomere damage (shortening), rendering the cell susceptible to apoptosis. L-NAME, by blocking both NOS pathways, protects against telomere damage and apoptosis (not depicted in the model).

specifically inhibits Hsp90 function, this is not an unexpected finding.

Interestingly, prostate cells are capable of recovering from RAD even after extensive telomere damage, since withdrawal from RAD restored telomere lengths to levels observed in untreated cells. This, in turn, suppresses the induction of apoptosis in these cells, thus demonstrating a connection between critically short telomeres and induction of apoptosis.

Further support for this model was demonstrated in our system by showing that the NOS inhibitor L-NAME was capable of eliminating free radical production and protecting cells from telomere shortening, directly implicating the source of the telomere damage as NOS-induced free radicals. The fact that telomerase is expressed in both RAD- and siRNA-treated cells further suggests that this is a telomerase-independent telomere-shortening mechanism. Interestingly, both conditions resulted in high levels of NOS-dependent free radical production, which provides convincing evidence that these two processes are directly linked.

The precise mechanism of how telomere shortening contributes to the onset of drug-induced apoptosis is not immediately clear. We speculate that while telomerase is functionally inhibited from its telomere maintenance mechanism, the telomere erosion observed might be due to multiple mechanisms that result in the deprotection of the telomere and hence telomere dysfunction. Disruption of its structure would likely render the telomere more susceptible to free radical-induced telomere damage, and this structural change could be the result of a disruption of telomere binding protein function. Additionally, because many DNA repair proteins are associated with the telomere (7), it is plausible to suggest that inhibition of Hsp90 may prevent these repair proteins from properly functioning, leading to a lack of telomere repair and an accumulation of ROS-induced telomere damage.

Free radicals have been demonstrated to be intimately involved in the induction of apoptosis, yet the mechanism(s) of free radical-induced apoptosis in response to different stimuli is poorly understood. While both free radicals and telomere shortening can induce apoptosis, we do not understand how these biologic processes interconnect. Consistent with our findings, an earlier study has demonstrated that the consequence of $^{\cdot}OH$ radical-induced apoptosis was telomere erosion (36), indicating a strong correlation between free radical-induced telomere shortening and apoptosis and adding additional support to our model.

Summary. Understanding the complex nature of DNA damage and apoptosis induced by Hsp90 inhibition, both genetically and pharmacologically, will contribute to improved use of Hsp90-inhibitory compounds therapeutically and may help to identify additional agents that are likely to work synergistically to increase specific killing of cancer cells. The work presented here has provided important insights into the consequences of Hsp90 inhibition on telomerase and telomere biology. While we demonstrate telomerase inhibition in prostate cancer cells by chaperone inhibitors in the short term, our data clearly indicate that telomerase inhibition cannot be maintained long term using these compounds, thus revealing that anti-Hsp90 compounds would be ineffective telomerase inhibitors. However, other aspects of Hsp90 inhibition may make these agents potentially useful as adjuvant therapies. Some Hsp90 inhibitors are currently in clinical trials and have been shown to simultaneously target multiple signaling pathways involved in promoting tumorigenicity. Here, we provide additional insights into the complex pharmacology of these agents with respect to the Hsp90 inhibitor radicicol. We demonstrate that RAD is capable of producing free radicals even at low concentrations, which in turn translates into specific free radical-induced telomere damage. Moreover, we find that long-term culture of cells with the Hsp90 inhibitor RAD results in delayed apoptosis as a consequence of critically short telomeres. Our knowledge of how different chemotherapeutic agents may induce DNA damage and apoptosis by free radical mechanisms may potentially lead to better treatment strategies and adjuvant therapies that can prevent cancer development and disease recurrence.

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